

# **The Gene Encoding Scrapie PrP Protein: Implications for the Nature of the Scrapie Agent**

INAUGURAL - DISSERTATION

ZUR  
ERLANGUNG DER PHILOSOPHISCHEN DOKTORWÜRDE  
VORGELEGT DER  
PHILOSOPHISCHEN FAKULTÄT II  
DER  
UNIVERSITÄT ZÜRICH

VON  
**BRUNO OESCH**  
von Balgach

BEGUTACHTET VON  
HERRN PROF. DR. C. WEISSMANN

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ZÜRICH 1986



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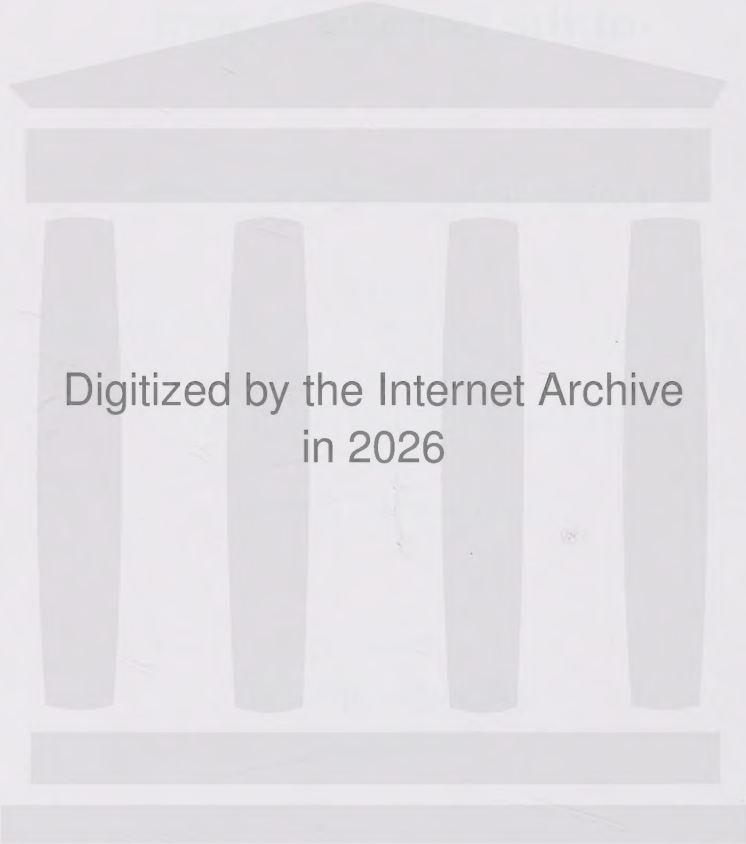
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# Für Terry und meine Eltern

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wo chiente mer hi  
wänn alli seite  
wo chiente mer hi  
und niemer giengti  
für einisch z'luege  
wohi dass me chiem  
we me gieng

Kurt Marti (1967)

### **Acknowledgment**

I wish to express my gratitude to Prof. Dr. C. Weissmann for his continuous advice, support and encouragement. His clear ideas proved to be essential at many stages of this thesis. Last but not least I also enjoyed his technical assistance in a few experiments.

I am also indebted to Prof. Dr. S. B. Prusiner and his group at the University of California in San Francisco for many stimulating discussions about scrapie and beyond. Their supply of biological material was vital for the work presented in this thesis.

Markus Aebi, Monika Wälchli and Koni Basler provided a very pleasant laboratory environment (D5) conducive to creative activities. I also profited many times from the experience and technical excellence of Werner Boll. Thanks are also due to Dani Schuppli for his excellent technical assistance.

Finally my thanks go to all members of the Institut für Molekularbiologie I for their suggestions, technical help and moral support.



## **Summary**

PrP 27-30 is the major protein in purified preparations of scrapie agent. Cognate cDNA clones were selected from scrapie-infected hamster brain cDNA libraries by oligonucleotide probes corresponding to the N-terminus of the protein. Southern blot analysis with PrP cDNA revealed a single gene present in the hamster genome. The same restriction pattern was observed for scrapie-infected and normal brain DNA. A single PrP-related gene was detected in murine and human DNA. Subsequently, hamster genomic clones corresponding to the PrP gene were isolated. The gene contains a noncoding 5' exon of 52 to 82 bp and a 2 kb coding exon, separated by a 10 kb intron. PrP-related mRNA was found at similar levels in normal and scrapie-infected brain, as well as in other normal tissues. Analysis of the 5' end of PrP RNA revealed multiple transcription initiation sites in vivo and in vitro. The PrP coding sequence encodes a 28 kd protein containing an N-terminal membrane insertion signal peptide. The primary structure of PrP encoded by the gene of a healthy animal does not differ from that encoded by a cDNA from an infected animal. The protease-resistance of PrP protein acquired upon infection with scrapie agent is therefore attributed to posttranslational events.

## **Introduction**

Scrapie is a transmissible, degenerative neurological disease of sheep and goats (Parry, 1983). The infectious agent is the best characterized member of a class of pathogens that cause spongiform encephalopathies in animals (transmissible mink encephalopathy, chronic wasting disease in elk and mule deer, scrapie; Hartsough and Burger, 1965; Williams and Young, 1982; Prusiner, 1982) and humans (kuru, Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome; Gajdusek, 1977; Masters et al., 1981).

### **Etiology of slow virus diseases**

The mode of transmission of natural scrapie is unknown. Goats fed scrapie-infected tissue frequently develop disease (Hadlow et al., 1974). In hamsters experimental transmission of scrapie by intracerebral inoculation is  $10^9$  times more effective than oral transmission (Prusiner, 1982). Epidemiologic studies of kuru in New Guinea provide evidence for ritualistic cannibalism to be involved in man-to-man transmission in the Fore population. A decline in incidence of kuru has been observed since the acculturation of the Fore people from a culture practicing endocannibalism to a modern society (Gajdusek, 1977). While kuru had a yearly incidence rate of about one percent and was localized to a small area in New Guinea, Creutzfeldt-Jacob disease is a rare, usually sporadic disease occurring worldwide. In about 10 percent of the cases a familial, autosomal dominant pattern of inheritance is observed (Gajdusek, 1977). Accidental man-to-man transmission of Creutzfeldt-Jakob disease by cornea transplantation or upon treatment with human growth hormone have been

reported (Duffy et al., 1974; Gibbs et al., 1985; Koch et al., 1985).

The incubation time of these diseases greatly varies between individual species or, within a species, between different genotypes (Gajdusek, 1977; Dickinson, 1976). The longest incubation time (up to decades) is found in humans for kuru (Prusiner et al., 1982b).

Over the past 20 years, scrapie has been extensively studied in laboratory rodents (Chandler, 1961); hamsters are the preferred experimental host because they have the shortest incubation periods (Marsh and Kimberlin, 1975). Intracerebral inoculation of hamsters with the scrapie agent is followed by an incubation period of 60 or more days before neurological symptoms appear, and death ensues 10-14 days later (Kimberlin and Walker, 1977; Prusiner et al., 1982a). In a given experimental system the incubation time is a logarithmic function of the inoculation dose in the range between  $10^3$ - $10^8$  ID<sub>50</sub>. This fact is used to determine the titer of a given sample (Prusiner et al., 1980). At high inoculation doses ( $>10^8$  ID<sub>50</sub> in hamsters) the incubation time approaches a minimum (50-60 days) which defines the shortest incubation time that can be observed for a given strain of animals and scrapie agent.

### **Genetic control of the incubation time of scrapie by the host**

Inoculation of different strains of mice with scrapie agent led to the identification of a host gene (*Sinc*) controlling the incubation time. By inoculation of different inbred mouse strains with the scrapie agent strain ME7, the alleles *Sinc*<sup>S7</sup> (short incubation time, 170 days in C57BL mice) and *Sinc*<sup>P7</sup> (prolonged incubation period, 340 days in VM mice) were defined. Heterozygous mice (s7p7) showed an incubation period midway between the parent strains showing no dominance for either allele.

However, a different strain of scrapie agent (22A) shows exactly the inverse behavior in the parental strains, i.e. longer incubation periods in s7 than in p7 homozygous mice. Unexpectedly, *Sinc* heterozygotes infected with strain 22A have longer incubation periods than the parental mouse strains, showing single-gene overdominance (Dickinson et al., 1968; Dickinson and Fraser, 1979). Apparently the phenotype of the *Sinc* alleles is dependent on the scrapie strain used for infection.

However, in mouse strains probably carrying the same *Sinc* allele differences in incubation period could be detected. A different gene (*PID*, prion incubation determinant) was mapped to the D subregion of the H-2 complex (Kingsbury et al., 1983). A series of alleles coding for short (q), intermediate (p, s, b, k) and longer (d) incubation periods were identified, the longer incubation period alleles appearing to be autosomally dominant.

A close linkage between a restriction fragment length polymorphism (RFLP) of the PrP gene and a gene controlling the incubation period of ME7 in NZW and ILN/J mice (*Prn-i*) was established (Carlson et al., 1986); the allele for the long (220 days) incubation time (*Prn-i<sup>b</sup>*) is autosomally dominant over the short (110 days) allele (*Prn-i<sup>a</sup>*). The relationship between the *Sinc* and the *Prn-i* locus is unknown; they may be identical. Transferring the cloned PrP gene of mice with the dominant long incubation allele (*Prn-i<sup>b</sup>*) to mice with the short allele (*Prn-i<sup>a</sup>*) should yield a long incubation period after infection if the PrP gene controls the incubation period.

### **Replication and pathology of scrapie**

The highest titers of scrapie agent are found in the brain, which is considered to be the main site of replication. Titers approximately 1000

fold lower were detected in spleen and other lymphoid tissues, while little or no infectivity was found in lung (Hadlow et al., 1974; Kimberlin and Walker, 1986). Although the ultimate target organ for scrapie is the central nervous system, the infectious agent appears to first replicate in the spleen after intraperitoneal inoculation. Increase of infectivity in hamster spleen is found early after i.p. infection, reaching a plateau prior to replication of the scrapie agent in the brain (Clarke and Haig, 1971). Splenectomy does not affect the incubation time indicating that replication in the spleen is not a necessary event for infection (Kimberlin and Walker, 1977, 1986). It may also be that the increase in infectivity in a particular organ is not due to local replication of the infectious agent but to transport and accumulation of infectious particles produced elsewhere.

The known pathological lesions of scrapie in hamster are confined to the nervous system and consists of vacuolation of neurons, astrogliosis, and amyloid plaques (Zlotnik and Rennie, 1965; Marsh and Kimberlin, 1975; Bendheim et al., 1984). Amyloid plaques are extracellular deposits of proteins which bind Congo red and thereupon exhibit green birefringence by polarisation microscopy. This property is attributed to domains with a high degree of beta structure (Glennner et al., 1972). It is known that various proteins can form amyloid (Puchtler et al., 1962). Amyloid plaques in scrapie-infected hamster brain as well as in human brains from patients with Creutzfeldt-Jakob disease or Gerstmann-Sträussler syndrome stain with antibodies to PrP. Amyloid plaques observed in other diseases like systemic amyloidosis in mice or Alzheimer's disease in humans do not show any immunoreactivity with antibodies to PrP (Barry et al., 1986; Kitamoto et al., 1986).

By negative stain electron microscopy filamentous structures specific for scrapie-infected brain have been identified in fractions of scrapie-infected mouse brain (scrapie-associated fibrils, SAF; Merz et al., 1983). Although they resemble amyloid they are morphologically distinguishable (Merz et al., 1983; Carp et al., 1985). The major constituent of purified SAF is a 26 kd protein (Diringer et al., 1983a) which is probably identical to PrP 27-30 (Rubenstein et al., 1986); PrP 27-30 was identified in amyloid plaques by immunocytochemistry (Bendheim et al., 1984; Barry et al., 1986). SAF have rarely been observed prior to detergent extraction (Rubenstein et al., 1986); it is known for PrP that it assembles into rods upon detergent extraction and represents an artefact of the detergent treatment (Meyer et al., 1986).

### **Characterisation of the infectious agent**

Unusual properties have been reported for the scrapie agent and for the other slow infectious pathogens (for a review see Prusiner, 1982). The scrapie agent is relatively resistant to boiling, and treatment with psoralens, formalin or hydroxylamine. Generally, the infectivity is unaffected by nucleic acid-inactivating agents but sensitive to reagents that inactivate or destroy proteins, such as SDS, guanidinium ions, urea, phenol or proteinase K (Prusiner, 1982). The scrapie agent is highly resistant to ultraviolet and ionizing radiation, behaving more like a protein than a virus or viroid (Bellinger-Kawahara et al., 1986). Calculations of the target size yield a molecular weight of the scrapie agent between 50,000 and 150,000 (Alper et al., 1966; Bellinger-Kawahara et al., 1986). Irradiation at 254, 267 and 280 nm generated essentially identical dose-response curves, which is unexpected

for a nucleic acid (Latarjet et al., 1970). Nucleic acids usually show maximal inactivation at 260 nm. These properties led to the new term prion, defined as a small *proteinacious infectious* particle which is resistant to inactivation by procedures that modify nucleic acids (Prusiner, 1982).

However, a different interpretation of the ionizing radiation data (Rohwer, 1984) suggests that the scrapie agent contains a nucleic acid with a possible size between  $5 \times 10^5$  daltons for a single-stranded and  $2 \times 10^6$  daltons for a double-stranded genome. The apparent existence of over 20 strains of scrapie agent in mice with respect to incubation time and lesion profile indicates the presence of an inheritable informational molecule in the infectious agent (Dickinson and Fraser, 1979; Bruce and Dickinson, 1979, 1986). These strains appear to have varying stabilities, resulting in mutation rates up to  $10^{-3}$  to  $10^{-4}$  for the most unstable strains (Dickinson, 1976; Dickinson and Outram, 1983). Some strains like 87A or 87V display discontinuous changes in their properties during passage in a single mouse genotype while other strains like 22A are stable in one mouse strain (VM) but not in others (C57BL). Only few scrapie strains are stable in more than one mouse genotype (Bruce and Dickinson, 1979). Since the host clearly influences the properties of the scrapie agent, it is difficult to compare strains other than those which are stable in the same mouse genotype. 22A and ME7 are both stable in VM mice and differ in their incubation time (200 days for 22A, 340 days for ME7; Bruce and Dickinson, 1979; Dickinson and Fraser, 1979). Since the host is the same, the difference in incubation time must be due to inheritable information contained in the infectious agent. Conventionally, genetic information would be stored in a nucleic acid, but one might also envisage

other possibilities, which will be analyzed in the Discussion.

### **Analysis of highly purified, infectious preparations of scrapie agent**

The purification of the scrapie agent from infected hamster brain by S. B. Prusiner and his colleagues marked a major advance in the field. The purification involved treatment with detergent, micrococcal nuclease, and proteinase K in the presence of sodium dodecyl sarcosinate (Sarcosyl), followed by sucrose gradient centrifugation. Fractions from the bottom of the gradient contained infective agent enriched from 3,000 to 10,000 fold with respect to protein. The rest of the gradient still contained considerable amounts of infectivity which appeared to be quite heterogeneous (Bolton et al., 1982; 1984). SDS polyacrylamide gel electrophoresis revealed the presence of a major protein component, designated PrP 27-30, detected only in preparations from infected brain but not in control preparations from normal brain (Bolton et al., 1982, 1984; Prusiner et al., 1983).

Electron microscopy of purified infectious preparations showed rod-shaped particles resembling purified amyloid, which when arranged in arrays showed green birefringence by polarisation microscopy after staining with Congo red dye (Prusiner et al., 1983). Further purification required the dissociation of these aggregates, and a variety of detergents was tested. Mild detergents were ineffective while denaturing detergents like SDS dissociated the aggregates but also abolished infectivity. SDS-denatured PrP 27-30 was then further purified by HPLC and its N-terminal sequence determined (Prusiner et al., 1984). A set of oligonucleotides based on reverse translation of the amino acid sequence

were synthesized, which I used to screen a cDNA library made with mRNA from infected hamster brain. It was believed that PrP would be encoded either by a viral genome or by a host gene activated upon infection. A clone encoding PrP 27-30 (this thesis; Oesch et al., 1985) was used to search for homologous sequences in the hamster genome. PrP-related sequences were present in the hamster genome and PrP mRNA was detected in normal as well as in scrapie-infected hamster brain; to reconcile the absence of PrP protein in the normal brain preparation one would have to invoke translational control of scrapie-specific PrP 27-30 expression. However, further analysis by S. B. Prusiner and coworkers revealed that the absence of PrP protein in the normal brain was due to the fact that it is protease-sensitive in the uninfected animal and escaped detection because the purification procedure involved protease digestion.

By Western blot analysis of crude extracts from both normal and scrapie-infected brain with monoclonal as well as polyclonal antibodies to PrP, proteins of 33,000 to 35,000 daltons were detected and designated PrP 33-35<sup>C</sup> and PrP33-35<sup>Sc</sup>, respectively (Oesch et al., 1985; Meyer et al., 1986; Barry et al., 1986). Upon limited proteinase K digestion, PrP 33-35<sup>Sc</sup> gave rise to PrP 27-30 in the case of scrapie-infected brain preparations, while PrP 33-35<sup>C</sup> in normal brain extracts was completely degraded (Bolton et al., 1984; Meyer et al., 1986; Barry et al., 1986). Aggregation of the proteins is a possible event leading to protease-resistance. However, infectious preparations of microsomes from scrapie-infected hamster brain did not contain rod-shaped aggregates, yet the <sup>3</sup>PrP isoforms showed the same properties as described above. It was only after detergent extraction of microsome preparations that rods were observed (Meyer et al., 1986).

The highest molecular weight form of PrP observed by Prusiner and coworkers is PrP 33-35. However, Bendheim and Bolton reported finding a 54 kd protein sharing antigenic epitopes with PrP in hamster and mouse brain. In normal brain extracts only the 54 kd protein was detected but not the lower molecular weight forms seen in infected brain; from this it was argued that the 54 kd protein is the precursor for the other PrP proteins (Bendheim and Bolton, 1986). Other investigators did not detect this protein on Western blots, neither with polyclonal nor monoclonal antibodies (Oesch et al., 1985; DeArmond et al., 1985). Its relationship to PrP therefore needs further clarification.

### **Outline of the work described in this thesis**

When I started to work on scrapie in 1982, the requirement of proteinacious component(s) was a well established fact based on the inactivation of the scrapie agent in crude or partially purified homogenates (Prusiner, 1982; McKinley et al., 1981; Prusiner et al., 1981). It was assumed that this protein component was either encoded by a putative genome of the infectious agent or a host gene induced upon infection.

Initially my project was to clone mRNAs specifically expressed in scrapie-infected brain. Such clones were thought to lead either directly to the infectious agent or at least would represent molecules important in the pathology of the disease. The identification of PrP27-30 as a scrapie-specific protein component and the determination of its N-terminal sequence led to the conclusion that cloning of PrP would be a more direct approach. Although it turned out that the scrapie-specific trait of PrP 27-30 is acquired posttranslationally, I spent some time

characterizing the gene and the corresponding mRNA. These studies may well be interesting but they will not yield the key to the nature of the scrapie agent. If the modified PrP protein is the scrapie agent, its genesis will be shown by protein biochemists. The more attractive hypothesis (for a molecular biologist) is that of an essential nucleic acid hidden in the infectious preparation. As a consequence I started to clone nucleic acids from highly purified infectious preparations. Previous work (M. Wälchli and C. Weissmann, personal communication) had shown that only minute amounts of nucleic acids, if any, are present in the most purified infectious preparations, however the analysis did not reach the detection limit of one molecule per infectious unit which is the lowest frequency at which any essential nucleic acid has to be present. Cloning represents a more sensitive method to search for nucleic acids in the infectious preparations and preliminary results will be presented.

## **Results**

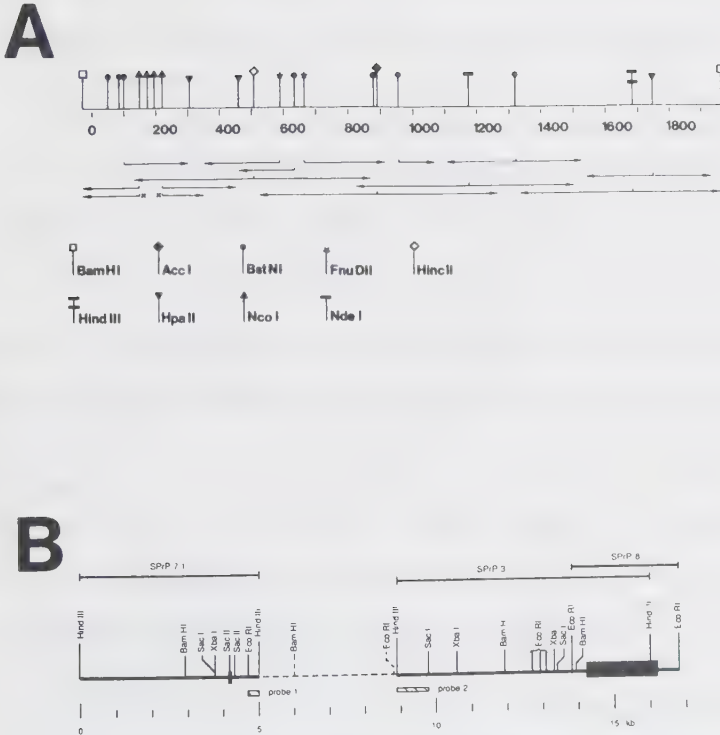
### **I. Studies on the PrP gene**

#### **Strategy**

Previous work done by S. B. Prusiner and coworkers suggested the scrapie-specific expression of PrP 27-30. The first approach to clone scrapie-specific mRNAs was by differential hybridisation of a cDNA library from scrapie-infected hamster brain using labeled cDNA from either infected or uninfected hamster brain as a probe. During the course of these experiments the N-terminal amino acid sequence of PrP 27-30 was elucidated prompting the synthesis of oligonucleotide probes in order to isolate the corresponding cDNA. Since it was unknown where the PrP protein is encoded, we assumed that an mRNA encoding PrP protein would be present in scrapie-infected brain during the exponential phase of scrapie agent replication, no matter whether it was encoded in the hamster genome or in a putative viral genome. For convenience we also assumed that it would be polyadenylated, as almost all mRNAs are thought to be.

#### **Cloning of PrP cDNA**

In the experimental system used, intracerebral inoculation of hamsters with  $10^7$  ID<sub>50</sub> units scrapie agent results in exponential increase of the titer between 10 and 40 days after inoculation, reaching a plateau after 50 days. Between 50 and 65 days after inoculation, the animals begin to develop signs of progressive neurological dysfunction, leading to death about 2 weeks later. Poly(A)<sup>+</sup> RNA was isolated from scrapie-infected hamster brain 35 and 45 days after inoculation in order to have RNA



**Figure 1: (A) Restriction Map of the HaPrPcDNA-1 Clone and Sequencing Strategy**

Origins of arrows indicate the restriction sites used for sequencing. The fragments were either labeled by 5'-phosphorylation with polynucleotide kinase or by filling up 5'-overhanging 3' termini with the Klenow fragment of DNA polymerase (x).

**(B) Genomic Organisation of the PrP Gene**

Exons are shown as black boxes. The three genomic clones used to characterize the gene are indicated at the top of the Figure. The dashed line represents an uncloned region which was partially mapped by Southern analysis using probe 1.

corresponding to the log and the late log phase of scrapie agent replication.

Poly(A)<sup>+</sup> RNA isolated from scrapie-infected hamster brain 35 days post inoculation was used to construct a cDNA library by the procedure of Okayama and Berg (1982); however a different vector was used (pHG327; Staeheli et al., 1986). A set of 32 icosameric oligonucleotides, 5'-GG(T/C)TT(A/G)TTCCA(T/C)TG(A/G)TT(A/G)TG, was synthesized, based on the reverse translation of a seven amino acid segment of PrP 27-30, N-His-Asn-Gln-Trp-Asn-Lys-Pro-C (Prusiner et al., 1984). Screening of 150,000 colonies with the 5'-[<sup>32</sup>P]-labeled probe mixture yielded a positive clone, from which the recombinant plasmid pHaPrPcDNA-1 was isolated.

### **The structure of the HaPrP cDNA**

The plasmid pHaPrPcDNA-1 contained an insert of about 2 kb. A provisional restriction map was established, and the insert was sequenced by the Maxam and Gilbert (1980) method. All regions were sequenced on both strands (except for two short stretches within the 3' noncoding region) and across the restriction sites that served as origin for the sequencing (Figure 1A). The heteropolymeric sequence comprised 1918 nucleotides (Figure 2A, residues 116-2032) and was preceded by 33 G residues and followed by 56 A residues. Primer extension experiments suggested that the pHaPrPcDNA-1 did not correspond to the full length RNA. Further cDNA was synthesized by M. Scott and D. Westaway in S. B. Prusiner's group and cloned in  $\lambda$ gt10 yielding an almost full length cDNA clone (pHaPrP-s11; Basler et al., 1986). The heteropolymeric sequence of pHaPrPcDNA-s11 was found to extend 114 nucleotides beyond the 5' end of pHaPrPcDNA-1. However, comparison of the sequence at the 5' end of clone

s11 with the genomic sequence, which was cloned later (see below), revealed that the first 33 nucleotides are due to a cloning artefact (Figure 2, nucleotides written in lower case letters; a detailed mechanism is described in Basler et al., 1986).

### **Identification of the cDNA sequence encoding PrP protein**

Numbering of the cDNA sequence starts at the first nucleotide of clone HaPrPcDNA-s11. The longest open reading frame extends from nucleotide 1 to 844 and the first in-frame ATG is found at position 83. A protein encoded by this reading frame would comprise 254 amino acids and have a molecular weight of 27,900. The major N-terminal amino acid sequence of PrP 27-30 (Prusiner et al., 1984) is encoded from nucleotide 350 to 394; the sequence of the cyanogen bromide peptides described in Oesch et al. (1985) also fit the predicted amino acid sequence starting at positions 419 and 500 (underlined in Figure 2A). This confirms that the cloned cDNA corresponds to PrP 27-30. The amino terminus of PrP 27-30, as isolated after proteinase K digestion, would be encoded from position 350 and could extend to position 844. If so, it would comprise 165 amino acids and have a molecular weight of 18,700. Sequencing of PrP 33-35<sup>Sc</sup>, obtained without protease digestion (Hope et al., 1986), showed a homogenous N-terminal amino acid sequence which is encoded in the cDNA starting at position 149 (N-Lys-Lys-Arg-Pro-Lys-; Figure 2A). A protein starting at this position would comprise 232 amino acids and have a molecular weight of 25,400.

### **PrP is encoded in the hamster genome**

Chromosomal DNA from normal brain of an outbred hamster was cleaved with EcoRI, BamHI or PstI, electrophoresed through an agarose gel,

**Figure 2: (A) Nucleotide Sequence of pHaPrPcDNA-s11 and pHaPrPcDNA-1**

The numbering of the nucleotides and amino acids starts at the first nucleotide and amino acid, respectively. The start of pHaPrPcDNA-1 and the position of exon joining are indicated. The underlined amino acids correspond to the peptides found by amino acid sequencing. Amino acid sequence A corresponds to the N-terminal sequence of PrP 27-30 (Prusiner et al., 1984). Sequences B and C correspond to internal peptides of PrP 27-30 described in Oesch et al. (1985). The underlined nucleotide sequences denote the putative polyadenylation signals.

**(B) Nucleotide Sequence of the PrP 5' Exon Region**

Numbering of nucleotides starts with the first nucleotide of the TaqI recognition site. The major transcription initiation start points deduced from S1 analysis are indicated by large arrowheads, and from primer extension by dots. The small arrowheads denote the transcription start sites for less abundant transcripts.

A

10 30 50  
 gccttcctcggctcgtcgtcgcgaacgcgcgcggggGAGAAGGCACATCGAGTCCACT  
 ← 5' exon → ← 3' exon → pHaPrP  
 70 90 110 cDNA-1  
 CGTCGCGTCGTTGGCAGATCAGCCATCATGGCGGAACCTTAGCTACTGGCTGCTGGCAGCT  
 METAlaAsnLeuSerTyrTrpLeuLeuAlaLeu  
 1 10  
 130 150 170  
 TTTCGGCTATGTGGACTGATGTTGGCCTCTGCAAGAAGCGGCCAAAGCCTGGAGGCTGG  
 PheValAlaMetTrpThrAspValGlyLeuCysLysLysArgProLysProGlyGlyTrp  
 20 30  
 190 210 230  
 AACACTGGCGGAAGCCGATACCTGGGCAGGGCAGCCCTGGAGGCAACCGTTACCCACCT  
 AsnThrGlyGlySerArgTyrProGlyGlnGlySerProGlyGlyAsnArgTyrProPro  
 40 50  
 250 270 290  
 CAGGGTCGCGCACATGGGGCAACCCCATGGTGGTGGCTGGGACAGCCCATGGTGGT  
 GlnGlyGlyGlyThrTrpGlyGlnProHisGlyGlyTrpGlyGlnProHisGlyGly  
 60 70  
 310 330 350  
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 80 90  
 370 390 410  
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 GlyGlyGlyThrHisAsnGlnTrpAsnLysProSerLysProLysThrAsnMetLysHis  
 100 110  
 430 450 470  
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 120 130  
 490 510 530  
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 SerAlaMetSerArgProMetMetHisPheGlyAsnAspTrpGluAspArgTyrTyrArg  
 140 150  
 550 570 590  
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 160 170  
 610 630 650  
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 TAAATACTGATGTGACCCTCTGACTTCCACACAGAGTACGCAGTGACAGACACACCTAAC  
 1210 1230 1250  
 TCTTAAATAGCGGAAGGGTTCTACAGCCAAAGAAGTCACTGTTTGGCATGGTCCCTAAG  
 1270 1290 1310  
 AAACAGCCTCCCATTTGGGATATTTAAAGCATCCATATGAGGCAATTCCTCCTTCACTAAC  
 1330 1350 1370  
 AAACCTCTAGCTGAGTAAGGCAACGGGAAAAAACAAAATTACCTACTAACATGGAAAGC  
 1390 1410 1430  
 AAACCTGTGTTCAATTTCTAGGAACTAGAATGATGTTTTAGCCTTGCTTGGAATGAACCA  
 1450 1470 1490  
 CGAGATTTTGGCTCTGAAGAGCCAACACTGTAAAAATGTGCTCCTCCTCGAAAGGGAGAG  
 1510 1530 1550  
 ATGGTTAGGACACAAAGTCACGGCGCTTGGCGTTTCTTCATTTCTGTCTCATAATTCTCA  
 1570 1590 1610  
 AAAGTCACAATTAGGTCATGCCCTTAGTTAATATATCTGTATTGAAATCGGACGACAAGA  
 1630 1650 1670  
 CACAATCTAAAAATTTCTCTAGGTTCTAGATGAAATAGGCTCCATTTCAAGGTGAAAGAC  
 1690 1710 1730  
 AGTTTCTTAGCGTTGCTTATGTAAGGCAAACTTGTTCTCCTTAAGTTGCTCCGTGTTTCCCT  
 1750 1770 1790  
 TGAGCAGACATAAACCCTCTGCAACAGCATTGCCCTGCTGTAGAATATATAAAGTGTAAC  
 1810 1830 1850  
 TACAAGCTTAGACCTTCTGTTCTGATGCATCCGAAGTACGTAATGCAGTACCATTTTCAC  
 1870 1890 1910  
 CCGGTATCAGATGTTTTCTGTGTGGCCCTAGCTTTCTTCAACATGCATTCCGTTCCAT  
 1930 1950 1970  
 ATATGAATCCAAAGTGGACCCCTAACTGGTCTCTGAAATCTGCATGTACTTTCACATTTT  
 1990 2010 2030  
 CTATATTTGTAACCTTGCATGTCCTTCTTTTGTCAATATAAAAGTTTATAAATGTTT(A)<sub>n</sub>

B

TCGAAATCTCCTCTTTAGCAATTTCTTGCTCTAGAGTTTCAGCAATGCTTCTCGCTCCATTAGGCAACCTTTTCATTTTCTCAGCT  
10 30 50 70 90

TCCCCATTATGTAACGGGAGCAATGGGTTCTGGACCACTCTTCGATTAAAGATGATTTTATAGTCGGTGAGCGCCGTCAGGGAGTGATG  
110 130 150 170

ACACTCGGGGCGGTTTAAACCGTAGAATCCCTTAACCAGTCTCGAGCGGTGACTCATTTCGCCAGGAGAAGTGGCGCGGCCAATTGGT  
190 210 230 250 270

GAGCAGGACGCAACCCCCCGGACCCAGCCCGCCCCCGGAGCTACCCCTCTGACTCACATCCCCCGCGCTCCCCCGCGGCTCCG  
290 310 330 350

AGCAGCAGACCGAGAAGGCACATCGAGTCCACTCGTCGCGCTCGGTGCGAGGTAAAGCGGCTTCTGAACCCCTGGCCCCGGGAAGGGTCTCGG  
370 390 410 430 450

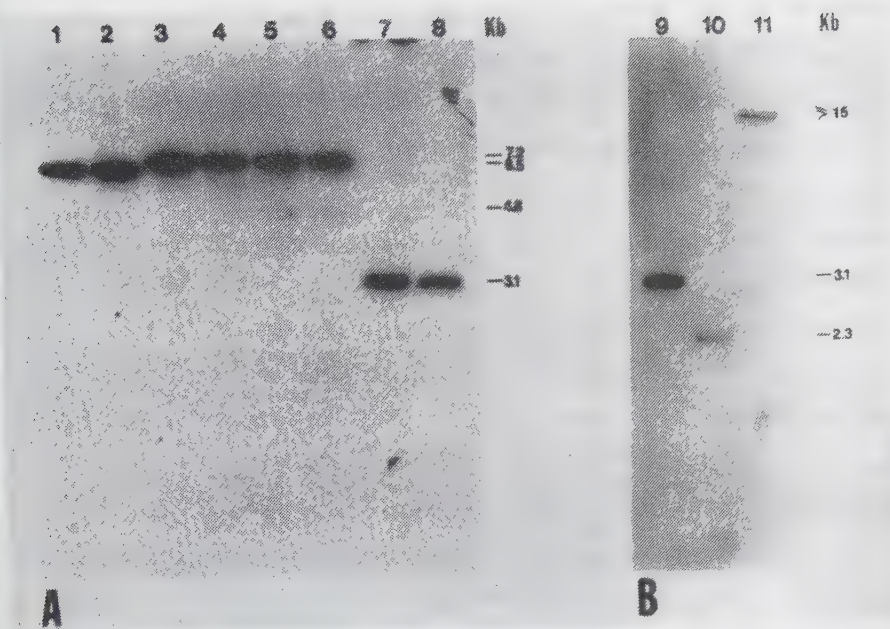
AGCCAGGCTCGGTAAGCCTTGGCTTCCAGAGCCAAAGCCCGGCTTACTCGGGCTCTCGGGGCGCTAGGCGCGGGGGCTGAGGTTGAG  
470 490 510 530

TCTGGCTGGGAGTGAACCGCCACCCGAGCGCGCGTCTCTTGAGGGACCGAAGCCAGGAGAGCCAGGAGCCATCCCTTCTCTCCG  
550 570 590 610 630

AGCCCGGCTACCCCCAGAGTCGTCGGGGATGGGGATGGGGATGGGGTGGCATCTTTTGACTGTCTTTGCTGTTTCTTCTCTCTT  
650 670 690 710

TGTAAAGCTACAGCGAACAATTTTACCAGGGTTCACCGTGGTCTCGTCGTCCTCGGCATCTCTCAGTCCAGTACATACCCAAAG  
730 750 770 790 810

and analyzed by Southern blotting (Southern, 1975), using the radiolabeled cDNA insert from pHaPrPcDNA-1 as a probe. Single bands at 3.1 kb, 6.5 kb and 2.6 kb were detected (data not shown). The same cleavage pattern was observed when chromosomal DNA from infected or uninfected hamster brain was analyzed by Southern blotting (Figure 3A, lanes 1 and 2 for BamHI, and lanes 7 and 8 for EcoRI; the experiment shown was done by D. Westaway in San Francisco). Two bands, a strong one at 7.2 kb and a weaker one at 4.8 kb, were generated by digestion with HindIII, an enzyme that cleaves within the HaPrPcDNA-1 clone. Further Southern analysis with the HaPrPcDNA-s11 clone indicated the presence of an additional exon since additional bands of 7 kb (for EcoRI) and 5 kb (for HindIII) were detected (K. Basler, personal communication). The 5' exon was then cloned in a 5 kb HindIII-HindIII fragment in  $\lambda$ 1149 using an oligonucleotide corresponding to the 5' end of HaPrPcDNA-s11 as a probe (Basler et al., 1986). To establish a linkage map between the genomic clones SPPr-7.1 (comprising the 5' exon) and SPPr-3/8 (containing the 3' exon; for genomic organisation see Figure 1B), and to assess the length of the intron, I searched by Southern analysis for single restriction fragments hybridizing to probes derived from the ends of the clones SPPr-7.1 and SPPr-3 (Figure 1B, probes 1 and 2). DNA fragments hybridizing to both probes would therefore span the uncloned intron region, and by knowing the position of the restriction sites in the cloned genomic DNA it would then be possible to estimate the length of the intron. The labeled RNA probes used were a 0.3 kb transcript upstream of the gap (Figure 1B, probe 1: EcoRI-HindIII of SPPr-7.1) and a 0.9 kb transcript downstream of the gap (probe 2: HindIII-SacI of SPPr-3). As shown in Figure 4 (Gel A), a 6.0 kb SacI and a >30 kb SacII fragment hybridized to probe 1. When probe 2 was hybridized to the same gel containing the SacI- and SacII-cleaved DNA, bands



### Figure 3: Southern Transfer Analysis of Genomic Sequences Related to pHaPrPcDNA-1

DNA samples (10 $\mu\text{g}$ ) were digested with restriction endonucleases, electrophoresed through a 0.9% agarose gel, transferred to a nitrocellulose filter and hybridized with  $^{32}\text{P}$ -labeled insert of pHaPrPcDNA-1 as described in Experimental Procedures.

(A) Southern blot analysis of brain DNA obtained from normal (lanes 1, 3, 5, 7) or scrapie-infected hamsters 75 days after inoculation (lanes 2, 4, 6, 8). Lanes 1 and 2, BamHI; lanes 3 and 4, BglII; lanes 5 and 6, HindIII; lanes 7 and 8, EcoRI.

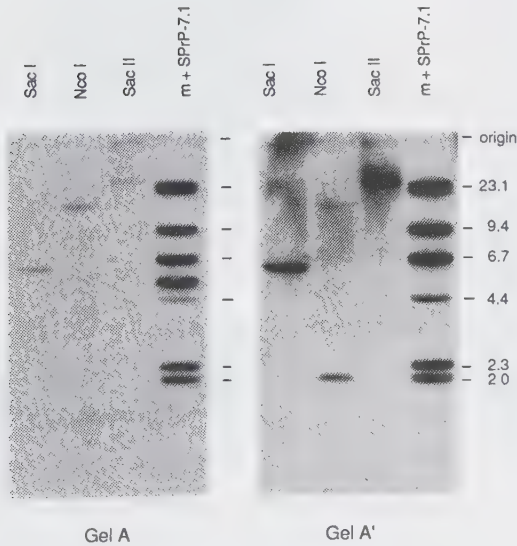
(B) Detection of pHaPrP-related sequences in normal mouse and human DNA. DNA samples were digested with EcoRI and processed as described above. Lane 9, hamster DNA; lane 10, mouse DNA; lane 11, human placental DNA. Sizes are indicated in kilobases. The filter was hybridized under conditions of reduced stringency.

appeared at the same positions as with probe 1 (Figure 4, Gel A'). Therefore, the distance between the *SacI* site of SPPr-7.1 and the most 5' *SacI* site of SPPr-3 is 6 kb, which means that a 3.9 kb segment is interposed between SPPr-7.1 and SPPr-3. The length of the intron is therefore 10 kb.

Southern analysis of murine and human genomic DNA cleaved with *EcoRI* revealed single bands of 2.3 and >15 kb, respectively, with HaPrPcDNA-1 as a probe (Figure 3B). No other bands were seen in the hamster DNA under the less stringent conditions used for the interspecies analysis. These results are compatible with a single PrP gene in the hamster genome. Furthermore, the detection of related sequences in murine and human DNA suggests that this gene has been conserved during mammalian evolution. In the meantime cDNA clones encoding the mouse and human PrP have been isolated and sequenced (Chesebro et al., 1985; Kretzschmar et al., 1986a; Liao et al., 1986).

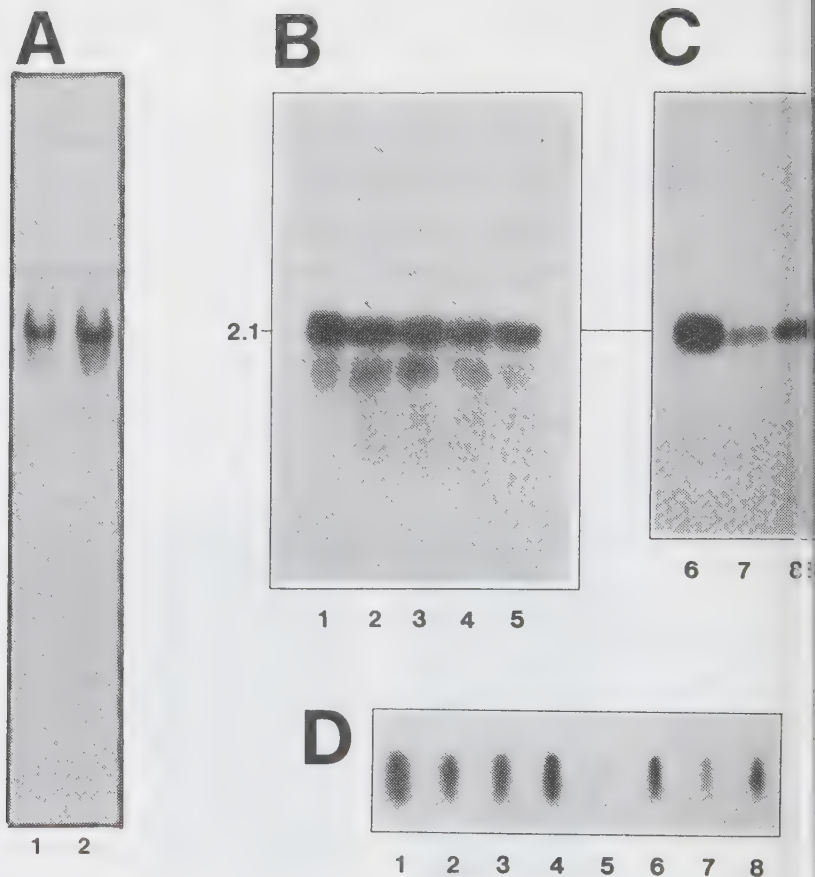
### **Detection of PrP mRNA transcripts**

Poly(A)<sup>+</sup> RNA prepared from brains of normal and scrapie-infected animals was electrophoresed through an agarose gel, and analyzed by Northern blotting (Thomas, 1980) using the insert of pHaPrPcDNA-1 as a probe. A major band of about 2.1 kb was found in infected as well as in uninfected samples (Figure 5A). Additional analyses done by D. Westaway revealed no significant quantitative differences between samples obtained at different times after infection, or between infected and control animals (Figure 5B). Polyadenylated RNA of a similar electrophoretic mobility was found in heart and lung of uninfected animals (Figure 5C). The lower molecular weight RNA species in Figure 5B are not a reproducible feature of these analyses and are attributed to mild degradation of the



#### Figure 4: Determination of the Intron Length by Southern A nalysis

Hamster DNA (5  $\mu$ g) was digested with the indicated restriction enzymes, electrophoresed through an agarose gel and analyzed by in situ hybridization with probe 1 (gel A, probes see Figure 2). After autoradiography gel A was alkali-treated as described in Experimental Procedures and rehybridized with probe 2 (Gel A'). The right hand lanes contain  $^{32}$ P-labeled HindIII cleaved  $\lambda$  DNA and 30 pg of the 5 kb HindIII fragment of SPrP-7.1. The absence of the SPrP-7.1 5 kb band in gel A' confirmed the proper removal of probe 1.



**Figure 5: Analysis of PrP mRNA from Normal and Scrapie-Infected Hamsters**

(A) Northern blot analysis of mRNA isolated from hamster brain 35 days after inoculation with either scrapie-infected (lane 1) or normal brain extract (lane 2). Poly(A)<sup>+</sup> mRNA (10 μg) was electrophoresed through a 1% agarose gel, transferred to nitrocellulose filters, and hybridized with <sup>32</sup>P-labeled cDNA insert of pHaPrPcDNA-1.

(B) Northern blot analysis of mRNA (10 μg) from uninoculated hamsters 40 days old (lane 1) or from scrapie-infected hamsters (lanes 2-5) 24 hr, 20 days, 40 days, and 60 days after inoculation, respectively.

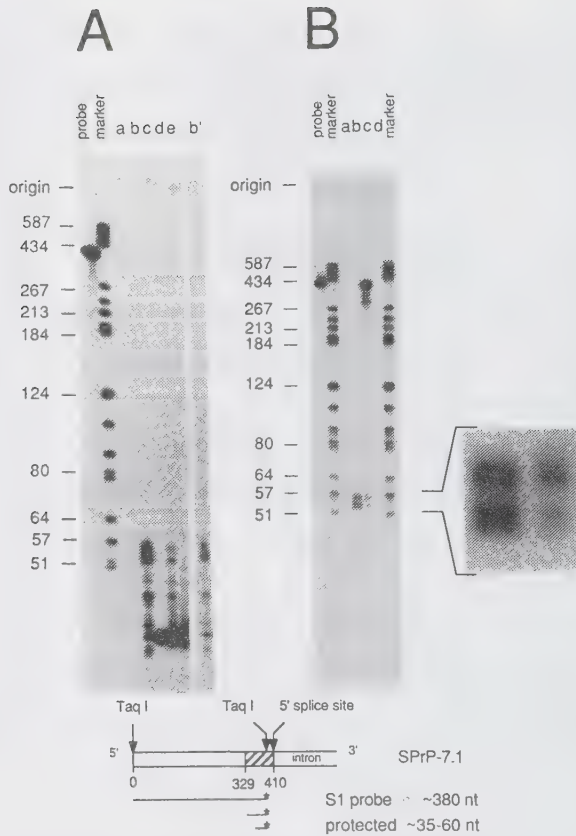
(C) Northern blot analysis of RNA (10 μg) from brain (lane 6), heart (lane 7), and lung (lane 8). Each sample contains 10 μg of poly(A)<sup>+</sup> RNA isolated from uninfected animals.

(D) Slot blot analysis of total RNA (25 μg) from various organs of 40-day-old uninoculated hamsters. Nitrocellulose filters were hybridized with the HaPrPcDNA-1. Slot 1, brain; 2, heart; 3, lung; 4, pancreas; 5, liver; 6, spleen; 7, testes; 8, kidney.

sample. Figure 5D shows by dot blot analysis that PrP-related transcripts were also detected in heart, lung, pancreas, spleen, testes, and kidney of uninfected animals. No reproducible hybridization signal was detected in liver RNA.

### **Mapping the 5' end of PrP mRNA**

To determine the 5' end of PrP mRNA, an S1 nuclease protection experiment was performed with the single-stranded probe indicated in Figure 6, using poly(A)<sup>+</sup> RNA of infected hamster brain. The protected fragments (Figure 6A, lane b and b') were heterogeneous in size. The most abundant 5' ends mapped to positions 331, 333, 334, 342 and 351 (Figure 2B). The 5' end of the longest fragment observed mapped to position 330. As S1 mapping identifies not only 5' ends of RNA but also mismatches between the RNA and the probe, primer extension was carried out in parallel to the S1 analysis, with a 35 nucleotide primer labeled at the same site as the S1 probe. Primer extension identifies 5' ends of RNA or stop sites for reverse transcriptase, due for example to secondary structure of the RNA. The S1 and reverse transcriptase products were run on the same gel (Figure 6A). The ends of the major primer elongation products mapped to positions 331, 334 and 342, i.e. the same positions as found in the S1 analysis (Figure 2B). Some of the less abundant 5' ends gave rise to bands of different relative intensities in the two experiments. These differences may be due to unequal sensitivity of the ends to S1 nuclease or preferential termination by reverse transcriptase. However, the overall band pattern was the same with both methods, suggesting strongly that authentic 5' termini of the RNA had been mapped.



**Figure 6: S1 Nuclease and Primer Elongation Analysis of the 5' End of PrP RNA**

(A) Poly(A)<sup>+</sup> RNA from infected hamster brain (lanes b, d and b') or yeast RNA (lanes a, e) was annealed to the strand-separated TaqI fragment for S1 analysis (lanes a, b and b') or to the primer (lanes d and e), processed as described in Experimental Procedures and autoradiographed 3 days (lane b') or 14 days (lanes a-e). Lane c, primer.

(B) S1 nuclease mapping of RNA from yeast (lane a), from scrapie-infected hamster brain (lane b), from SPPr-7.1 transcribed in vitro as described in Experimental Procedures (lane c) and from HeLa cell nuclear extract only (lane d) was done with the same probe as in (A). The area of the major transcription start sites between position 330 and 334 is enlarged at the right. The structure of the 5' end of the gene is shown at the bottom of the Figure. The 5' exon is hatched. The asterisk at the 5' end of the probe denotes the position of the <sup>32</sup>P label.

### **In vitro transcription of the PrP gene**

Although 5' ends are indicative of transcriptional starts, they may also come about by specific cleavage of the RNA. Therefore, SPrP-7.1 DNA was tested as a template for in vitro transcription, using HeLa cell nuclear extract (Dignam et al., 1983). Transcription was performed on a supercoiled template containing the 5' exon and the putative promoter region in the presence of non-radioactive nucleoside triphosphates and the resulting RNA was analyzed by the S1 nuclease protection assay with the probe indicated in Figure 6. The protected fragments (Figure 6B, lane c) show the same heterogeneity of transcription initiation sites as the RNA from hamster brain (Figure 6B, lane b). To examine the relative strength of the PrP gene promoter, the Adeno major late promoter was transcribed in parallel under the same conditions. S1 mapping of the transcripts indicated that the Adeno major late promoter is approximately ten fold stronger than the PrP promoter (data not shown).

## **II. Cloning of nucleic acids from highly purified infectious preparations**

### **Strategy**

As outlined in the Introduction, different strains of scrapie agent have been described, of which at least some are genetically stable suggesting the presence of an inheritable informational molecule in the infectious agent. If it were a nucleic acid, it should be possible to clone it. Although no nucleic acid has been detected in purified infectious preparations of scrapie agent, the analytical methods may not have been sensitive enough to reveal as little as one copy of an essential nucleic acid per infectious unit. Nonetheless, it should be possible to detect such a nucleic acid by molecular cloning. By using the most highly purified infectious preparation

as a starting material, contaminations by normal hamster nucleic acids would be minimized. Since the type and form of nucleic acid is unknown, heat denaturation of the preparation followed by double strand cDNA synthesis with random oligonucleotides as primers was chosen as the most promising approach. By this strategy linear and circular single-strand (ss) RNA as well as single-stranded and double-stranded (ds) linear DNA would be converted into double-stranded linear DNA suitable for cloning. Other forms such as ds RNA or ds circular DNA may not be cloned, as it is uncertain whether ds RNA will be denatured under the conditions used and denaturation of ds circular DNA will yield form IV DNA, a form which probably does not permit cloning any more. Further complications arise by the fact that, if only small amounts of nucleic acids are present, these might be lost during deproteinization and therefore addition of carrier nucleic acid is required. In vitro synthesized globin RNA was chosen as a carrier nucleic acid in order to allow its identification and elimination after the cloning. Additionally, the carrier served as a standard for cDNA synthesis and cloning efficiency (see below).

### **Isolation and cloning of nucleic acids from infectious preparations**

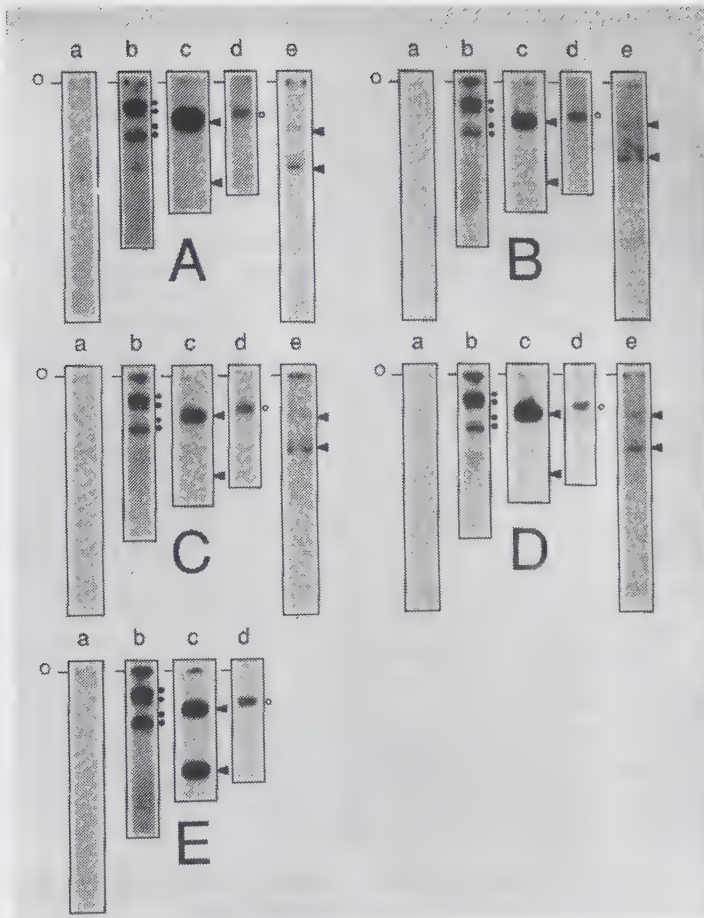
Nucleic acids from  $10^8$  infectious units of scrapie agent were purified in the presence of 50 ng in vitro synthesized  $\beta$ -globin pre-mRNA containing the three exons, the first intron and a truncated second exon (for details see Experimental Procedures). The length of the  $\beta$ -globin RNA was 800 nucleotides; 50 ng therefore correspond to  $10^{11}$  molecules. The ratio of scrapie-specific to  $\beta$ -globin clones was expected to be at least  $1:10^3$ . The purified nucleic acids were reverse transcribed with random

oligonucleotides as primers and cloned after EcoRI linker addition in  $\lambda$ gt11 (Young and Davis, 1983) which was chosen for its high cloning efficiency (for details see Experimental Procedures). In the initial cloning approximately 20,000 independent recombinants were obtained and screened by in situ hybridization with the globin probe. 90% of the clones contained globin-related sequences. The rest of the clones may contain small  $\beta$ -globin inserts which do not hybridize, EcoRI linkers or nucleic acids derived from the infectious preparation. To recover inserts of 100 bp or greater I amplified the library as a plate lysate, isolated the  $\lambda$  DNA, cut out the inserts with EcoRI and, after size selection by agarose gel electrophoresis, recloned them into  $\lambda$ gt11/amp3 (Kemp et al., 1983). 10,000 clones of the secondary library were then screened for the presence of  $\beta$ -globin related sequences which were found in more than 99% of the clones. 0.3% of the clones showed development of blue color indicative for an intact  $\beta$ -galactosidase and therefore represent clones without insert. White plaques not hybridizing to the  $\beta$ -globin probe were present at a frequency of about 0.5%; they were picked and further analyzed; these may contain the desired "scrapie DNA", hamster RNA-derived cDNA, genomic DNA which contaminated the purified PrP preparation, or E. coli DNA which was not removed completely prior to the recloning of the  $\lambda$  inserts. Since the EcoRI cloning site was located in a 2.2 kb MluI-MluI  $\lambda$  DNA fragment, the shift of this fragment to higher molecular weight was used to estimate the size of the inserts. MluI-MluI fragments containing an insert of more than 100 bp were isolated, nick-translated and hybridized to separate strips of Southern blots carrying either hamster or E. coli LE 392 genomic DNA, both cleaved with EcoRI,  $\beta$ -globin DNA or 4 genome equivalents of  $\lambda$ gt11/amp3 DNA cleaved

with MluI. These hybridizations would therefore allow further elimination of undesired clones.

48 clones were picked and 33 were analyzed for the size of their insert. 22 had an insert clearly longer than 100 bp and the MluI-MluI fragment was isolated. 18 MluI-fragments were used for hybridization to Southern blots. Four clones hybridized neither to hamster, *E. coli* or globin DNA (Figure 7A-D, lanes a, b and c). The strips containing the  $\lambda$  DNA demonstrate that under these conditions homologous sequences in the hamster genomic DNA would have been detected (Figure 7, lanes d). As a control, the Southern blots containing the hamster DNA were rehybridized with HaPrPcDNA-s11; a 3 kb band for the 3' exon and a 7 kb band for the 5' exon were revealed (Figure 7, lanes e). The bands appearing in the Southern blot with *E. coli* DNA are probably due to hybridization of the  $\beta$ -galactosidase sequences in the MluI fragment of  $\lambda$ gt11 DNA to the homologous *E. coli* segment. The fragment from  $\lambda$ gt11/amp3 gave the same signals (data not shown). The Southern blots carrying the globin sequences (Figure 7A-D, lanes c) did not hybridize (lower arrowheads) whereas the vector sequences (pSP64, upper arrowheads) hybridized, as pSP64 contains  $\beta$ -galactosidase sequences (Melton et al., 1984). Figure 7E shows an example of a clone containing globin-related sequences, as indicated by the lower band in lane c.

The 4 clones shown in Figure 7A-D are now under further investigation, i.e. the inserts are subcloned into pSP64 and will then be used to probe dot blots of the infectious preparation for the presence of related sequences at a frequency of at least one copy per infectious unit expected for an essential nucleic acid.



**Figure 7: Southern Analysis of Cloned Nucleic Acids From Infectious Preparations**

Nick translated *Mlu*I-*Mlu*I fragments of  $\lambda$ gt11/*amp*<sup>3</sup> clones 5.5 (A), 6.2 (B), 6.6 (C), 6.9 (D) and 6.1 (E) were hybridized to Southern blots of *Eco*RI cleaved DNA from hamster (5  $\mu$ g, lanes a) or *E. coli* LE 392 (3.3  $\mu$ g, lanes b), R3G/LIVS24 DNA inserted in pSP64 (10 ng) cut with *Hind*III, *Bam*HI, and *Eco*RI (lanes c) and  $\lambda$ gt11/*amp*<sup>3</sup> DNA (80 pg) cleaved with *Mlu*I (lanes d) as described in Experimental Procedures. Hamster genomic Southern blots of lanes a were rehybridized with nick translated pHaPrPcDNA-s11 (lanes e) except for clone 6.1 (panel E). Filled (lanes b) and open circles (lanes d) denote the hybridization signals due to  $\lambda$  sequences present in the *Mlu*I-*Mlu*I fragment. Arrowheads in lanes c indicate the position for pSP64 (upper) and globin sequences (lower); arrowheads in lanes e indicate the position of the signals for the 5' exon (upper; 7 kb) and 3' exon (lower; 3 kb).

## **Discussion**

For more than four decades the infectious agent causing scrapie has resisted elucidation. Development of an incubation time interval assay (Prusiner et al., 1980) facilitated the purification of the infectious particles (Prusiner et al., 1981, 1982a, 1983; Diringer et al., 1983a, 1983b). Examination of such preparations revealed a 27,000 to 30,000 dalton protein, named PrP 27-30, as being the most abundant macromolecule (Bolton et al., 1982, 1984). Inactivation studies on crude brain extracts suggested a protein component essential for scrapie infectivity (Millson et al., 1976; Prusiner et al., 1981) and this finding held also true for the purified agent (Bolton et al., 1982, 1984; Prusiner et al., 1982b, 1983; Diener et al., 1982; McKinley et al., 1983a; Diringer et al., 1983b). The N-terminal sequence of PrP 27-30 was determined (Prusiner et al., 1984), allowing the identification of cognate PrP 27-30 cDNA clones which in turn were used to investigate the relationship of PrP 27-30 and the scrapie agent, in particular the origin and structure of PrP 27-30.

### **PrP is encoded in the hamster genome**

The finding that PrP 27-30 was encoded by an RNA eliminated the possibility of protein-directed protein synthesis but still allowed for it to be encoded by a putative nucleic acid in the infectious particle. The gene should then be present at a frequency of at least one copy per infectious unit. Hybridisation of purified infectious preparations established the absence of PrP-related sequences, ruling out the possibility that PrP is encoded by the infectious agent (Oesch et al., 1985).

Southern blotting revealed the presence of the PrP gene in both,

infected and uninfected hamster genomic DNA (Figure 3). Single copy sequences hybridizing to the hamster PrP cDNA were also detected in chromosomal DNA of other mammals like human, goat, sheep, rat, and mouse as well as in invertebrates like nematode, drosophila, and possibly yeast (Figure 3B; Westaway and Prusiner, 1986) suggesting an evolutionary conservation. However, further experiments are required to show that cross-hybridization is due to homology in all cases.

Comparison of a cloned human and mouse PrP cDNA to the hamster PrP cDNA reveals for both approximately 90% homology on the protein as well as on the nucleotide level (Kretzschmar et al., 1986a; Chesebro et al., 1985).

Subsequently the hamster genomic gene was cloned and shown to contain a short 5' noncoding exon and a 2 kb protein coding exon, separated by a 10 kb intron (K. Basler, diploma work 1986; Basler et al., 1986; Figure 1B). S1 mapping and primer extension of PrP mRNA indicated the existence of multiple transcription start sites which mapped within a stretch of about 25 nucleotides (Figure 6 and 2B). In vitro transcription of the cloned genomic DNA encompassing the putative promoter and 5' exon gave rise to products whose 5' termini mapped to the same transcription initiation start sites as found in vivo (Figure 6B). The promotor region lacks a TATA box and contains three repeats of the sequence GCGCGCGC (Figure 2B; Basler et al., 1986), which resemble the Sp1 binding site (Dyran and Tjian, 1983) found in viral (Barrera-Saldana et al., 1985) and cellular (Dyran et al., 1986) promoters.

The occurrence of multiple start sites in the case of promoters lacking a TATA box has been described for a number of mRNAs, in particular SV40 late mRNAs (Gosh et al., 1978) and mRNAs for the 3-hydroxy-3- methylglutaryl coenzyme A reductase (Reynolds et al.,

1985), Thy-1 membrane glycoprotein (Giguère et al., 1985), adenosine deaminase (Valerio et al., 1985), c-Ha-ras proto-oncogene (Ishii et al., 1985a) and epidermal growth factor receptor (Ishii et al., 1985b).

### **The PrP gene is expressed in normal and scrapie-infected tissues**

PrP mRNAs with the same electrophoretic mobility were found in normal brain, heart, and lung as well as in scrapie-infected hamster brain (Figure 5). Related sequences were also present in total RNA from pancreas, spleen, kidney, and to a lesser extent also in testes and liver. Scrapie infectivity is found throughout the animal reaching the highest titers in the brain. This finding is compatible with the notion that PrP mRNA is ubiquitously expressed. However, other investigators were not able to detect PrP mRNA in spleen (Chesebro et al., 1985; Robakis et al., 1986), an organ implicated in the initial replication of the scrapie agent (Clarke and Haig, 1971). Scrapie-associated fibrils (SAF) had been detected in infected spleen (Merz et al., 1983; Rubenstein et al., 1986) and, in order to explain the absence of the PrP mRNA, it was proposed that SAFs were transported there at an advanced stage of the disease (Robakis et al., 1986). In view of the results presented here, namely the presence of PrP mRNA in spleen, there is no need for such an explanation.

The open reading frame determined for the PrP cDNA (Figure 2A) is uninterrupted between the multiple initiation sites and the first TGA at position 845 of the cDNA sequence. The first in-frame ATG is found at position 83, a few nucleotides downstream of the intron/exon junction. A second ATG occurs 42 nucleotides downstream. Most likely the first ATG corresponds to the initiation codon, because as a rule the most upstream ATG within a reading frame is used for initiation (Kozak,

1984a) and because the flanking positions (CCATCATGG) conform well to the consensus sequence (CCACCATGG) described for eukaryotic initiation sites (Kozak, 1984a, 1984b). Additionally, there is evidence that PrP<sup>33-35</sup><sup>Sc</sup> starts at amino acid position 23 (Hope et al., 1986; Figure 2A) indicating that translation starts at the first ATG (position 83, Figure 2A) and subsequent processing of the signal peptide results in the protein with the observed N-terminus (Bazan et al., 1986). Translation starting at the second ATG (position 125) does not lead to a functional signal sequence which would result in a protein with a different N-terminus (V. Lingappa, personal communication). The first in-frame stop codon upstream of the ATG triplet is at position 325 of the genomic sequence (Figure 2B), 4 residues upstream of the most 5' transcription initiation site observed.

The polyadenylation site at position 2032 is preceded by the sequence TATAAA at positions 2011 and 2022 (Figure 2A) which resembles the consensus sequence AATAAA usually found 11 to 30 residues upstream of the polyadenylation site of most eukaryotic mRNAs. Deviations from the consensus sequence have been described for the chick alpha actin gene (ATTAAA, Fornwald et al., 1982), the Ha-ras oncogene (AGTAAA, Capon et al., 1983), the mouse alpha amylase gene (AATAAA and AATATA, Tosi et al., 1981) or the hepatitis B surface antigen gene (TATAAA, Simonsen and Levinson, 1983). The hepatitis B gene uses the identical signal as proposed for the PrP gene.

### **Structure of the PrP precursor protein as deduced from the nucleotide sequence and its relationship to PrP molecules found in vivo**

The protein encoded by the 3' exon from positions 83 to 844 comprises

254 amino acids and has a calculated molecular weight of 27,921. At least part of the difference between the predicted and the observed molecular weight (33-35 kd) of PrP 33-35 is due to glycosylation (Bolton et al., 1985). There are two potential N-glycosylation sites of the type Asn-X-Thr, at codons 181-183 and 197-199.

While PrP has a unique primary structure, its secondary structure shares some interesting similarity to serum amyloid A proteins (Bazan et al., 1986). These acute phase proteins are the precursors of the insoluble, fibril-forming amyloid A proteins. The 12 kd precursor is cleaved at the C-terminus to yield a 8.6 kd protein which is deposited as amyloid during chronic inflammatory diseases (Yamamoto and Migita, 1985).

Computer analysis shows that PrP contains a stretch of hydrophobic amino acids near the N-terminus which have the structure of a signal peptide (Basler et al., 1986; Bazan et al., 1986). Three structurally dissimilar regions can be recognized which are typical for signal peptides (von Heijne, 1985; Perlman and Halvorson, 1983): the often positively charged N-terminal region of variable length (amino acids 1-6), a central hydrophobic region (h-region, amino acids 7-17), and the C-terminal region of 5 amino acids that precedes the cleavage site for the signal peptide. At the h/c region there is usually a charged amino acid, and the beginning of the mature protein is also predominantly marked by a polar residue. Therefore the proposed cleavage occurs at position 22/23. In fact, PrP 33-35<sup>Sc</sup> isolated from scrapie-infected hamster brain starts at position 23 (Hope et al., 1986) in perfect agreement with the predicted N-terminus of the mature protein. After chemical deglycosylation PrP 33-35 has a molecular weight of 25,000. The difference in molecular weight between the predicted precursor (27,921) and the deglycosylated PrP 33-35 is close to that predicted for the 22 amino acid N-terminal

signal peptide (2,500; S. B. Prusiner, personal communication).

In vitro translation of synthetic PrP RNA yielded a 28 kd protein in good agreement with the calculated molecular weight for the precursor (27,921). Addition of dog pancreas microsomes to the in vitro translation system resulted in the appearance of two more PrP-related proteins: a 25 kd protein presumably results from cleavage of the N-terminal 22 amino acid signal peptide and a 32 kd protein which may be a glycosylated form of PrP. In the absence of the first 14 amino acids, membrane insertion does not take place (B. Hay, S. B. Prusiner and V. Lingappa, unpublished observation). These findings support the predictions made from the nucleotide sequence, in particular that the PrP precursor molecule contains a membrane insertion signal peptide.

Digestion of PrP 33-35<sup>Sc</sup> with proteinase K yields the protease resistant core protein PrP 27-30 (Oesch et al., 1985; Meyer et al., 1986; Barry et al., 1986). Alignment of the N-terminal amino acid sequence (Prusiner et al., 1984) to the predicted sequence indicates that PrP 27-30 has lost about 67 amino acids (residues 23-89) from its amino terminus. Comparison of the amino acid composition of PrP 27-30 with that calculated from the translated sequence suggests that truncation may also occur at the C-terminus. There was reasonable agreement for most amino acids with the exception of serine, which was 78% too high, phenylalanine, which was 38% too high, and tyrosine and glycine, both of which were about 25% too low. Inspection of the 19 C-terminal amino acids of the predicted PrP sequence showed that they comprised a disproportionately high number of phenylalanine and serine residues—three of each—and no tyrosine and glycine residues. The amino acid composition deduced for a protein of 146 residues extending from position 350 to 787 was in much better agreement with the analytical

Table 1. Amino Acid Composition of Scrapie PrP 27-30

| Amino Acid | Determined by<br>Hydrolysis <sup>a</sup><br>Residues/Molecule | Predicted from cDNA <sup>b</sup><br>Residues/Molecule |
|------------|---------------------------------------------------------------|-------------------------------------------------------|
| Ala        | 8.5                                                           | 9                                                     |
| Arg        | 7.7                                                           | 8                                                     |
| Asn        | 16.6                                                          | 13                                                    |
| Asp        |                                                               | 6                                                     |
| Cys        | ND                                                            | 2                                                     |
| Glu        | 16.7                                                          | 7                                                     |
| Gln        |                                                               | 10                                                    |
| Gly        | 16.1                                                          | 14                                                    |
| His        | 5.2                                                           | 5                                                     |
| Ile        | 5.3                                                           | 4                                                     |
| Leu        | 3.9                                                           | 3                                                     |
| Lys        | 9.5                                                           | 8                                                     |
| Met        | 7.9                                                           | 9                                                     |
| Phe        | 3.2                                                           | 3                                                     |
| Pro        | 4.9                                                           | 5                                                     |
| Ser        | 5.9                                                           | 6                                                     |
| Thr        | 10.7                                                          | 12                                                    |
| Trp        | ND                                                            | 2                                                     |
| Tyr        | 11.4                                                          | 10                                                    |
| Val        | 8.5                                                           | 10                                                    |

<sup>a</sup>Average of four determinations based on 142 amino acids for PrP 27-30. Cys and Trp residues not included. Numbers taken from Oesch et al., 1985. ND: not determined

<sup>b</sup>Calculated from 146 amino acid residues, 90-235 in Figure 2A.

values (Table 1). PrP 27-30 may therefore, as a result of the proteinase K treatment used in the purification procedure, be truncated at both termini.

A striking feature of the predicted amino acid sequence is the occurrence of repeated sequences between codons 34 and 91 (Table 2), where two small repeats of GG(N/S)RYP are followed by a longer set of five repeats of P(H/Q)GGG(-/T)WGQ. These repeats are also conserved in the human PrP protein except for one substitution at amino acid position 56 (Figure 2A, Table 2; Kretzschmar et al., 1986a). The high degree of conservation suggests that they may be important for the normal function of the PrP protein. However, these sequences do not seem to be required for infectivity, as they are not present in PrP 27-30.

Interestingly, a 50 kd keratin protein that polymerizes into intermediate filaments has eight repeats of the tetrapeptide -Gly-Gly-Gly-X- within its N-terminal segment where -X- is a hydrophobic amino acid (Marchuk et al., 1984). In the case of the PrP protein, the amino acid at position -X- is a tryptophan, which is also hydrophobic, in four out of five repeated tetrapeptides (Table 2).

### **What is the difference between PrP isoforms from normal and scrapie-infected hamster brain ?**

When examined by Western blotting, extracts of both normal and scrapie-infected hamster brains contain PrP 33-35 (Oesch et al., 1985). Treatment of such brain extracts with proteinase K resulted in the disappearance of PrP 33-35, however, in scrapie-infected samples new proteins appeared corresponding in molecular weight to PrP 27-30. Further analysis of the protease-sensitive (PrP 33-35<sup>C</sup>) and of the resistant (PrP 33-35<sup>Sc</sup>) isoforms showed that PrP 33-35<sup>C</sup> is solubilized

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Table 2. Repeated Sequences in PrP cDNA

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|      |                                     |      |
|------|-------------------------------------|------|
| 182  | GGc GGa AgC CGa TAC CCT             | 199  |
| (34) | GLY GLY Ser ARG TYR PRO             | (39) |
| 215  | GGa GGc AaC CGt TAC CCa             | 232  |
| (45) | GLY GLY Asn ARG TYR PRO             | (50) |
| 233  | CtC CAg GGT GGc GGC aca TGG GGg CAa | 259  |
| (51) | PRO Gln GLY GLY GLY Thr TRP GLY GLN | (59) |
| 146  | CCC CAT GGT GGT GGC - TGG GGa CAG   | 283  |
| (60) | PRO HIS GLY GLY GLY - TRP GLY GLN   | (67) |
| 284  | CCC CAT GGT GGT GGC - TGG GGa CAG   | 307  |
| (68) | PRO HIS GLY GLY GLY - TRP GLY GLN   | (75) |
| 194  | CCC CAT GGT GGT GGC - TGG GGt CAG   | 331  |
| (76) | PRO HIS GLY GLY GLY - TRP GLY GLN   | (83) |
| 332  | CCC CAT GGT GGT GGC - TGG GGt CAa   | 355  |
| (84) | PRO HIS GLY GLY GLY - TRP GLY GLN   | (91) |

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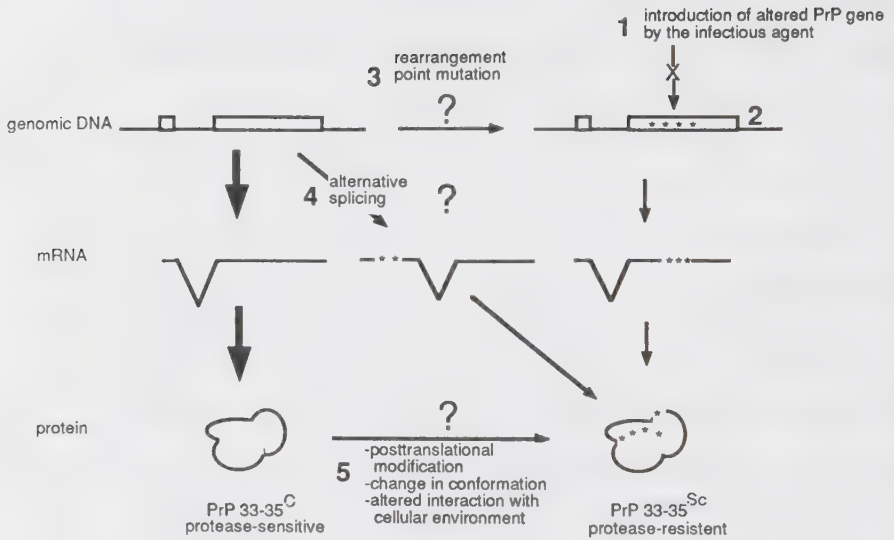
The consensus sequences are in uppercase letters, the deviant residues in lowercase letters.

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in detergent, while PrP 33-35<sup>Sc</sup> aggregates under the same conditions (Meyer et al., 1986). However, protease-resistance of PrP 33-35<sup>Sc</sup> does not seem to be an artefact of detergent extraction/aggregation since in native brain microsomes both isoforms retain their characteristic properties in the absence of any aggregation products (Meyer et al., 1986).

The different properties of cellular and scrapie PrP proteins may be due to chemical structure i.e. altered amino acid sequences or posttranslational modifications. Differences in amino acid sequences could come about if the PrP proteins were encoded by two different genes either (1) newly introduced by the infectious agent or (2) already present in the hamster genome, (3) by alteration of the coding sequence of the genomic PrP gene i.e. rearrangement or introduction of point mutations, (4) by alternative splicing of the PrP mRNA as illustrated in Figure 8.

However, these possibilities seem most unlikely for the following reasons: (1) no PrP-related sequences were detected in purified infectious preparations (Oesch et al., 1985) eliminating the possibility of the introduction of an altered cellular gene as in the case of oncogenes. (2) no evidence was found for two different cellular PrP genes by Southern blot analysis with a variety of restriction enzymes or under conditions of reduced stringency (Figure 3; Basler et al., 1986). The observed band pattern could always be explained by a single gene. (3) No difference was observed between normal or scrapie-infected DNA by Southern analysis (Figure 3A; Basler et al., 1986) which indicates that the PrP gene is not rearranged upon infection. However, if a rearranged PrP gene were present in only a small portion of the brain cells, it would have escaped detection. Although major alterations of the genomic PrP gene seem most unlikely, two genes differing only by point mutations would not necessarily have been detected by Southern analysis.



### Figure 8: Pathways to Produce PrP 33-35<sup>Sc</sup>

(1) Introduction of a modified PrP gene by the infectious agent or (2) the induction of a second genomic PrP gene would lead to the expression of a modified RNA and result in an altered PrP protein. (3) An altered PrP gene could also come about by rearrangement of the genomic PrP gene or introduction of point mutations. (4) Alternative splicing would result in the addition of coding sequence at the 5' end of the RNA resulting in a longer polypeptide chain. (5) PrP 33-35<sup>Sc</sup> or its precursor would be subject to posttranslational events resulting in PrP 33-35<sup>Sc</sup>. For more details see text.

Comparison of the protein-encoding region of two independent cDNA clones (pHaPrPcDNA-1 and pHaPrPcDNA-s11; Oesch et al., 1985; M. Wälchli, personal communication) derived from mRNA of scrapie-infected brain and that of the genomic gene isolated from normal hamster DNA (Basler et al., 1986) revealed no differences. However, as scrapie-infected brain contains both forms of PrP (Barry et al., 1986), there is no assurance that the cDNA clones isolated would be representative for PrP 33-35<sup>Sc</sup>. (4) Modification of the coding sequence could also occur by alternative splicing. This could come about if a conjectural exon comprising an open reading frame and an ATG initiation codon would be located further upstream of the 3' exon which contains the protein-encoding sequence. After splicing, the first ATG would be used for initiation of translation, resulting in a longer polypeptide chain than that of PrP 33-35<sup>C</sup>. However, both PrP isoforms have the same mobility on SDS PAGE (Oesch et al., 1985; Barry et al., 1986) and show the same reactivity towards polyclonal, affinity-purified antisera (Oesch et al., 1985), monoclonal antibodies against PrP 27-30, as well as antisera raised against synthetic peptides corresponding to residues 90-102 and 220-233 (Barry et al., 1986; R. Barry and S. B. Prusiner, personal communication). In addition, there is no difference in the Northern analysis of poly(A)<sup>+</sup> mRNA from normal or scrapie-infected animals (Figure 5; Chesebro et al., 1985; Robakis et al., 1986). A 54 kd protein crossreacting with antibodies against PrP was identified by Bendheim and Bolton (1986), however, its relationship to PrP has yet to be clarified as other investigators did not detect it (Oesch et al., 1985; DeArmond et al., 1985).

Since both PrP isoforms are glycosylated (Bolton et al., 1985; S. B. Prusiner, personal communication), differences in the sequence of sugar

residues or their site of attachment might explain the different properties of the two proteins (Figure 8, point 5). Numerous other posttranslational modifications of proteins such as phosphorylation, sulfatation, adenylation or covalent attachment of lipids, to mention but a few, have been observed. Whether these or other modifications account for the different properties remains to be determined. To date, PrP 33-35<sup>C</sup> has not been isolated which is a prerequisite for comparative studies of the two proteins.

Another model is that PrP 33-35<sup>C</sup> and PrP 33-35<sup>Sc</sup> are chemically identical but have different conformations and consequently interact differently with the cellular environment. A further possibility is that cellular components might be responsible for the properties of the PrP isoforms; for example, an infected cell may lack proteins PrP normally interacts with, or new proteins or nucleic acids may appear upon infection and bind to PrP 33-35<sup>C</sup> converting it into PrP 33-35<sup>Sc</sup>. A precedent for such a mechanism is found in hybridoma cell lines in which the production of immunoglobulin heavy chains in the absence of light chains appears to be deleterious to the cell (Köhler, 1980). An in vivo example are thalassemias caused by the unbalanced expression of  $\alpha$ -globin in excess over  $\beta$ -globin or vice versa (Collins and Weissman, 1986).

### **Search for nucleic acids present in purified infectious preparations**

To date, it is not convincingly shown that the infectious agent in fact lacks an essential nucleic acid component. Experiments designed to detect nucleic acids specific for the infectious agent directly (Marsh et

al., 1974; Manuelidis and Manuelidis, 1981; K. Gilles, D. Riesner, and S. B. Prusiner, personal communication) or by inactivation studies (Diener et al., 1982; McKinley et al., 1983a) have so far been unsuccessful. However, there is also evidence which suggests a possible role for nucleic acid in the infectious particle such as the existence of different strains of scrapie agent (Dickinson and Fraser, 1979; Bruce and Dickinson, 1979) or enhanced sensitivity of infectivity to UV irradiation in the presence of nucleic acid intercalating agents (Dees et al., 1985).

As the level of the conjectured scrapie nucleic acid may be too low for chemical detection, I am continuing the search by cloning the nucleic acids present in infectious preparations. Any nucleic acid essential for infectivity must be present at a frequency of at least one copy per infectious unit. I started with  $10^8$  ID<sub>50</sub> units mixed with  $10^{11}$  molecules globin RNA. Therefore the ratio of scrapie-specific clones to globin clones was expected to be  $10^{-3}$ , or higher, if for each infectious unit there is more than one copy of nucleic acid. In the primary library approximately 20 scrapie-specific clones can be expected. In 3000 globin clones of the secondary library 4 clones were identified which do not hybridize to hamster DNA, E. coli DNA, or globin DNA, yet should have done so if they had homologous sequences as judged by the positive controls (Figure 7, lanes d and e). However, these clones could still originate from other sources of contamination and, it remains to be shown that they specifically hybridize to nucleic acids derived from infectious preparations and not from control preparations. These experiments are in progress and will be reported elsewhere.

### Models for the scrapie agent and its replication

Biochemical and genetic evidence argues that the infectious scrapie agent must contain a protein and an inheritable, i.e. replicatable informational molecule, which may or may not be the same molecular entity. The critical genetic evidence is the demonstration of distinct strains of scrapie agent (for example, 22A and ME7), which breed true in one and the same host (in the case mentioned, the mouse strain VM) (Bruce and Dickinson, 1979, 1986; Dickinson and Fraser, 1979), and of apparent mutational events, which in certain host genotypes lead to what is interpreted as the selection of new scrapie strains (Bruce and Dickinson, 1986).

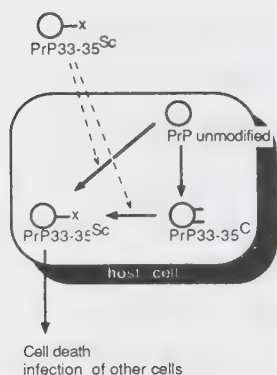
The major biochemical arguments that a protein plays an essential role in the transmission of scrapie are that proteolysis and protein-modifying agents reduce the infectivity of the infectious agent preparations, while exhaustive treatment with nucleolytic agents (nucleases,  $Zn^{++}$ ) do not (Millson et al., 1976; Prusiner, 1982; Diener et al., 1982); these findings definitively eliminate a viroid-like entity as the infectious agent (Prusiner, 1982; Diener et al., 1982). The presence of PrP 27-30<sup>Sc</sup> as the major component in highly purified scrapie agent preparations (Bolton et al., 1982, 1984), the finding that the concentration of PrP 27-30<sup>Sc</sup> was proportional to the scrapie agent titer, and the correlation between the kinetics of PrP protein degradation and of titer diminution (McKinley et al., 1983b) has led to the proposal that PrP 27-30<sup>Sc</sup> plays a causal role in the transmission of scrapie. The close genetic linkage of a restriction fragment length polymorphism of the PrP gene and a locus controlling the incubation time (*Prn-i*; Carlson et al., 1986; this locus may be identical with the *sinc* locus described by Dickinson and his colleagues (Dickinson et al., 1968; Dickinson and Fraser,

1979)) suggests that the PrP gene and the incubation time gene may be identical and provides further evidence for a role of PrP protein in scrapie. While the requirement for a protein is generally accepted, it is not formally proven that this protein is PrP, rather than a minor component which has not yet been detected, nor is it clear whether or not there are other components in the infectious particle. Moreover, it is possible that the role of the *Prn-i/PrP* gene product(s) is not at the level of scrapie transmission, but at that of pathogenesis, that is, the conversion of  $\text{PrP}^{\text{C}}$  to  $\text{PrP}^{\text{Sc}}$  may be a consequence of the infection, and the symptomatology of the disease may depend on the rate of the conversion and the precise properties of  $\text{PrP}^{\text{Sc}}$ , which may be different in the different host strains.

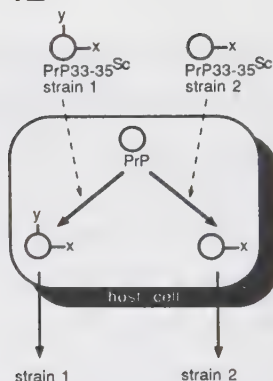
**Model for an infectious particle devoid of nucleic acid.** To date,  $\text{PrP}^{\text{Sc}}$  is the only characterized macromolecule found in purified preparations of scrapie agent.  $\text{PrP}^{\text{Sc}}$  is not found in normal brain but is closely related, and in all likelihood identical at the amino acid sequence level, with  $\text{PrP}^{\text{C}}$ , which is found both in normal and scrapie-infected brain. As outlined above, the different properties of the two forms of PrP are probably due to differences in posttranslational modification or in conformation.

For the purpose of the discussion in this section I shall assume that  $\text{PrP}^{\text{Sc}}$  is, by itself, the infectious scrapie agent; by this I understand that the introduction of  $\text{PrP}^{\text{Sc}}$  elicits (a) the pathological processes characteristic for scrapie and (b) gives rise to more  $\text{PrP}^{\text{Sc}}$  than had been introduced into the host. A further assumption is that there is only a single chromosomal gene for PrP, as appears likely from currently available evidence (Oesch et al., 1985; Chesebro et al., 1985; Basler et al., 1986; Westaway and Prusiner, 1986; note however, that an episomal form

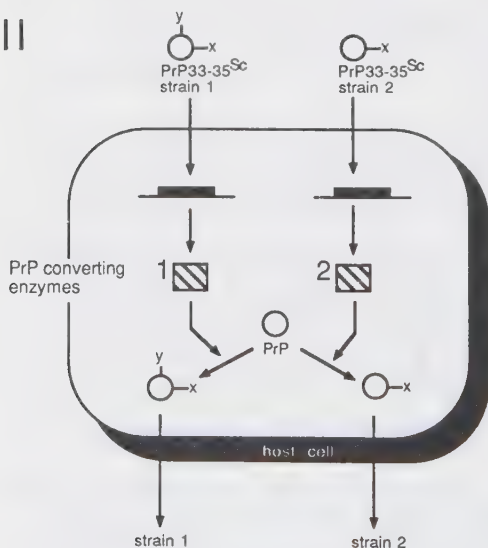
IA



IB



II



**Figure 9: Models for the Replication of Scrapie Agent Without a Nucleic Acid Component**

PrP 33-35<sup>Sc</sup> is the infectious agent. (I) "Direct action" model. Upon infection PrP 33-35<sup>Sc</sup> directs its own production from either the unmodified PrP or from PrP 33-35<sup>Sc</sup> (IA). The replication of different strains of PrP 33-35<sup>Sc</sup> in the same host is explained by assuming that strain specificity resides in different modifications (x,y) which in turn direct the same modifications (IB). (II) "Indirect action" model. Modified PrP activates cellular genes which convert cellular PrP protein into PrP 33-35<sup>Sc</sup>. Different modifications activate genes of different "PrP converting enzymes".

of the PrP gene has not been searched for, and may have escaped detection, particularly if it occurred only in a small proportion of brain cells) and that this gene is expressed to equal extents in normal and diseased animals (Oesch et al., 1985; Chesebro et al., 1985; Kretzschmar et al., 1986b; Robakis et al., 1986), giving rise to the same primary translation product. Under these assumptions one has to conclude that host-encoded, non-pathogenic PrP 33-35<sup>C</sup> or its precursor is converted into the modified, infectious form as a consequence of the introduction of PrP 33-35<sup>Sc</sup> ( or PrP 27-30<sup>Sc</sup>) into the organism. How could this transformation be triggered? The action of PrP<sup>Sc</sup> could be direct or indirect. In the case of direct action, PrP<sup>Sc</sup> would act like an enzyme, and convert either PrP 33-35<sup>C</sup> or its precursor into PrP 33-35<sup>Sc</sup> which in turn would catalyze further conversion (Figure 9A). As pointed out by Brunori and Talbot (1985), this process may be analogous to the conversion of trypsinogen to trypsin; one may visualize this model by considering a hypothetical disease, trypsinosis, which would occur in an imaginary animal in whose blood a stable trypsinogen circulates. The trypsinogen remains inactive, and the animal remains healthy, until it is inoculated with a trace of trypsin, whereupon trypsinogen is activated to trypsin, autolysis sets in and leads to the animal's death. Trypsinosis can be transmitted to other animals naturally, say by cannibalism, or experimentally, by inoculation with blood from a trypsinotic animal. In due course, investigators will isolate trypsin and identify it as the infectious agent, by demonstrating that it conforms, *mutatis mutandis*, to Koch's four postulates.

In an indirect mode of action, conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> would be catalyzed by a protein distinct from PrP<sup>Sc</sup> which does not occur in the uninfected cell. PrP<sup>Sc</sup> would initiate synthesis of more PrP<sup>Sc</sup> by

generating active "PrP converting enzyme", by action on either the transcriptional or some posttranscriptional level. One might even envisage PrP<sup>Sc</sup> initiating a cascade of events, which at some level would lead to activation of the converting enzyme.

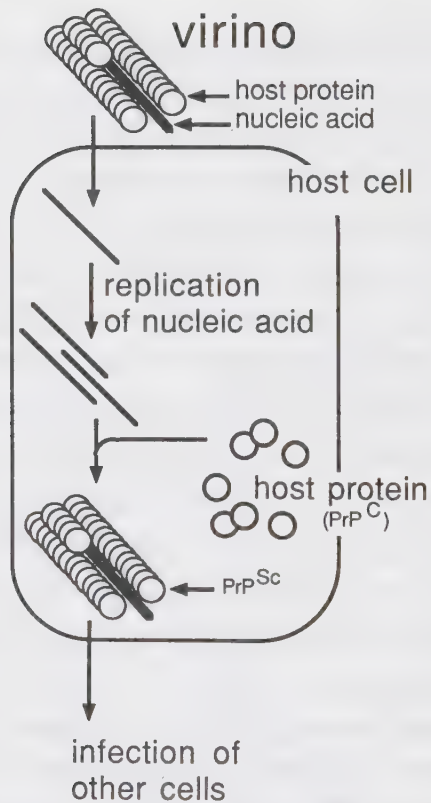
How can one account for distinct, stable strains of scrapie agent such as 22A or ME7 within the framework of the hypotheses outlined in this section? In the "direct action" model, each strain of scrapie agent would be represented by a different PrP<sup>Sc</sup> molecule, and it would have to modify a common precursor (because the presence of only one PrP gene in the inbred host is assumed) in such a fashion that in each case the product resembles the type of PrP<sup>Sc</sup> catalyzing the conversion (Figure 9B). There is no precedent for this type of reaction, and the greater the number of discrete scrapie strains that are capable of being stably transmitted by an inbred host, the less credible the model. The conversion of one scrapie strain to another upon change of host (or in a host heterozygous for the PrP gene) can be accommodated if the incoming PrP<sup>Sc</sup> of one type can convert the resident PrP precursor of another type, and if the same modification on a different PrP polypeptide would give rise to a new strain. At least in some cases, the properties of a strain would therefore depend on the type of modification as well as the PrP polypeptide. An example for strain conversion is provided by strain 22A, which breeds true in the VM mouse but is converted to strain 22F in the C57BL mouse (Bruce and Dickinson, 1979). It would be interesting to know whether passage of 22F in VM mice again would give rise to 22A. However, special pleading is required to account for the finding that the scrapie strain ME7 is stably transmitted in both the VM and the C57BL mouse.

Within the framework of the "indirect action" model, the variety of scrapie species would have to be explained by a battery of distinct

converting enzymes, each of which would convert one and the same PrP precursor to a different type of PrP<sup>Sc</sup> and would be specifically activated by that particular type of PrP<sup>Sc</sup>. The set of converting enzymes could differ in different host species, accounting for the "mutation" of some PrP strains upon changing the host.

How can these questions be tackled experimentally? The most direct approach is a biochemical one, and would aim at converting PrP<sup>C</sup> or PrP precursor (which could be synthesized in a cell free system) into infectious scrapie agent with use of scrapie brain cell extracts. The technical difficulty with this approach lies in the fact that any newly generated scrapie infectivity has to be distinguished from the large amounts of scrapie agent present in the cell extracts themselves; this problem might be overcome by using PrP precursor of one scrapie type and extracts derived from brains infected with another type. A genetic approach could be envisaged, in which for example a VM mouse containing a C57BL PrP gene (introduced by exhaustive back-crossing, or better, by transgenic methods) is infected with scrapie strain 22A, and the nature of the resulting scrapie agent is determined; if the strain 22F were generated, this would show that the PrP gene determines the strain of the scrapie agent. Needless to say, these are very demanding experiments and negative outcomes do not provide any further insight.

**Models involving a nucleic acid as the inheritable informational molecule.** The existence of distinct scrapie strains which undergo what appears to be mutation and selection (Bruce and Dickinson, 1986) is most easily explained by the assumption that the scrapie agent comprises a nucleic acid of some sort. However, despite considerable efforts, it has so far not been possible to detect a nucleic acid in the most highly purified preparations of infectious scrapie



**Figure 10: Model for the Scrapie Agent Containing a Nucleic Acid (Virino Model)**

A nucleic acid with an envelope consisting of host protein (virino) infects the cell. After replication of the nucleic acid, cellular PrP (PrP<sup>C</sup>) is assembled into the virino structure. In the most simple scenario conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> is caused by the association with the virino nucleic acid.

preparations (Marsh et al., 1974; K. Gilles and D. Riesner, personal communication; M. Wälchli and C. Weissmann, personal communication), and UV inactivation data have not provided support for the existence of a nucleic acid genome of more than 30-45 nucleotides (this target size was calculated assuming that the scrapie nucleic acid is double-stranded and readily repairable after UV damage; Bellinger-Kawahara et al., 1986). Nevertheless, a small nucleic acid, present at a level commensurate with that of infectious particles, and protected against nucleolytic agents in some fashion, has not yet been ruled out. The example of the coconut cadang-cadang viroid, an RNA of approximately 250 nucleotides, which causes profound pathological changes and the ultimate death of its host (Haseloff et al., 1982), provides dramatic evidence for the fact that length is not essential for potency.

Dickinson and his colleagues have proposed earlier (Dickinson and Outram, 1979; Kimberlin, 1982) ) that the scrapie agent consists of a scrapie-specific nucleic acid coated by a host-specified protein, a particle they designated as virino, and Oesch et al. (1985) have remarked that PrP<sup>Sc</sup> would be a likely candidate for such a host-specified coat protein. The PrP-virino model would explain many properties of the scrapie agent. The existence of different strains and the occurrence of mutations could be ascribed to variations in the small nucleic acid, just as in the case of viroids (Schnölzer et al., 1985); reproducible changes of scrapie agent strains upon passage in a certain host could be explained by mutation followed by selection (Bruce and Dickinson, 1986). The conclusion that a scrapie-specific nucleic acid has to be quite small and could not encode a protein of substantial size, and the absence PrP-encoding nucleic acid from preparations of scrapie agent are accounted for by the fact that PrP is a host-encoded protein. Last but not

least, the striking finding that the scrapie agent is not immunogenic and that the disease is not accompanied by any inflammatory reactions (McFarlin et al., 1971; Kasper et al., 1981), is explained by the tolerance of the host to a slightly modified host-specific protein. Within the framework of the PrP-virino model the conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> could be associated with the recruitment of PrP from a host cell membrane into the virino particle (Figure 10).

Although considerable evidence argues in favor of PrP protein being a component of the infectious agent, it is nonetheless possible that the active scrapie agent is a conventional virus which, when associated with PrP protein or rods, is unusually stable. As argued elsewhere, such a virus would have to have a ratio of infectious to total particles close to 1 in order to account for its not having been detected physically in highly purified preparations of scrapie agent (Oesch et al., 1985). The association of the virus with PrP proteins might facilitate infection and mediate the observed resistance to inactivating agents. If only a fraction of the virus were associated with PrP and if this subpopulation were much more infectious than the rest, purification using infectivity as an assay would lead to enrichment of PrP associated with the conjectured virus.

## Conclusions

As outlined above, it is currently not possible to settle on a model that satisfactorily explains all the properties attributed to the scrapie agent. Taken at face value, the UV inactivation data, the failure to find a nucleic acid component in highly purified preparations of infectious scrapie agent and the immunological data argue against the scrapie agent being a conventional virus. If one accepts the possibility that a small

scrapie-specific nucleic acid may so far have escaped detection, the virino hypothesis gains credibility. If the absence of any nucleic acid in scrapie agent preparations is accepted, then the existence of scrapie strains that breed true in one and the same inbred host strain and the mutational events that appear to modify scrapie strains lead to explanations which are not easily accepted in the light of conventional wisdom. At present, the preference of one model over another is based more on belief than of scientific facts. The mystery of the nature of the scrapie agent persists and will continue to stimulate the brains and pens of scientists.

## EXPERIMENTAL PROCEDURES

**Preparation of cDNA library.** Hamsters were sacrificed at various times after intracerebral inoculation with scrapie agent or extract of normal brain. Total RNA was prepared from brain tissue by the LiCl/urea method (Auffray and Rougeon, 1980). Poly(A)<sup>+</sup> RNA was selected on oligo(dT) cellulose (Aviv and Leder, 1972) and cloned essentially as described by Okayama and Berg (1982), using, however, a different vector, pHG327 (a gift of W. Boll; Stäheli et al., 1985). The difference is that cloning is carried out at an Sst I site, which is closely flanked by two Bam HI sites; this permits convenient excision of the cloned sequence. Transformation was into *E. coli* DH1 cells by the method of Hanahan (1983). One microgram of RNA gave rise to  $6 \times 10^4$  transformants.

**Screening the cDNA library.** Transformed *E. coli* DH1 ( $10^4$  per plate of 13 cm diameter) were grown on LB agar containing 100 µg/ml ampicillin. Filters for colony hybridization were prepared by the method of Taub and Thompson (1982). The oligonucleotide mixture was 5'-<sup>32</sup>P-labeled (Wallace et al., 1979) to a specific activity of  $6-8 \times 10^6$  cpm/pmol. Hybridisation was performed in 6 x NET (NET is 0.15 M NaCl; 15 mM Tris-HCl, pH 7.5; 1 mM EDTA), 0.5 % Nonidet P40 (NP40), 200 µg/ml yeast carrier RNA (Sigma grade VI) at  $7 \times 10^5$  cpm/ml for 16 hr at 42°C. The filters were washed in 6 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 47°C and exposed to Fuji RX films at -70°C with intensifier screens. Positive colonies were picked and recloned.

**Restriction site mapping.** The DNA fragments to be analyzed were end-labeled with <sup>32</sup>P, cleaved with restriction enzymes to yield two fragments and then isolated. Labeled fragments ( $1-2 \times 10^4$  cpm) were mixed with 0.5 µg carrier DNA (pHaPrPcDNA-1 or pSP64) and digested with 1 new unit (amount of enzyme that cleaves 1 µg of λ DNA in 15 min; Biolabs) of restriction enzyme for 2, 4, 8, and 15 min. The digested samples were run on a 1.2 % agarose gel, the gel dried and autoradiographed, and the fragment sizes determined relative to

markers.

**Nucleotide sequence determination.** Restriction fragments were 5'-<sup>32</sup>P-labeled with polynucleotide kinase or 3'-<sup>32</sup>P-labeled with the Klenow fragment of DNA polymerase I, cleaved with an appropriate restriction enzyme and sequenced by the Maxam and Gilbert (1980) procedure.

**Isolation of hamster DNA.** Golden Syrian hamsters (LVG/LAK) were purchased from Charles River Laboratories. Weanling hamsters were inoculated with 10<sup>7</sup> ID<sub>50</sub> units and sacrificed 70 days later. DNA from their brains as well as from uninfected controls was isolated by a proteinase K procedure (Gross-Bellard et al., 1973), as described by Payne et al. (1981) and used for Southern analysis. Alternatively, liver DNA was isolated from outbred Golden hamsters as described by Mantei et al. (1979). The DNA was >50 kb, as judged by electrophoresis on a 0.4 % agarose gel, using λ DNA as a molecular weight standard.

**Southern analysis.** Method A: Conventional transfer to nitrocellulose filters. Hybridization was performed with a randomly primed radiolabeled probe (Taylor et al., 1976; Payne et al., 1981) at 42°C in 3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 30% deionized formamide, 0.05 M HEPES (pH 7.4), 0.1% SDS, 0.1% sodium pyrophosphate, 100 µg/ml salmon sperm DNA and 200 µg/ml heparin (Sigma, sodium salt grade II; Singh and Jones, 1984) for 18 h. The filter was washed in 0.1 x SSC, 0.1% SDS at 65°C for 2 h, dried and autoradiographed at -70°C using a Dupont Cronex intensifying screen and Kodak XAR-5 film.

Method B: Hybridization of DNA immobilized in agarose gels using SP6 transcripts as probes (Purello and Balasz, 1983; Kidd et al., 1983; as modified by B. Wieringa, personal communication): DNA samples (5 µg) were digested with restriction endonucleases and run through a 0.7 % agarose gel. After incubation in 0.4 M NaOH, 0.8 M NaCl at room temperature and neutralization in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl at 4°C (30 min each) the gel was dried on a slab gel dryer at 60°C

between two sheets of Whatman 3MM chromatography paper, then wetted in water and the paper peeled off. The gel was prehybridized at 65°C for 1 to 2 h in 0.5 M sodium phosphate buffer (pH 7.2), 7 % (w/v) SDS (Biorad 101-0301) containing 25 µg/ml yeast carrier RNA. Hybridization was for 16 h at 65°C in a fresh batch of the same solution containing  $1.5 \times 10^6$  cpm/ml  $^{32}\text{P}$ -labeled SP6 RNA probe ( $10^8$  cpm/µg) prepared as described by Melton et al. (1984). The gel was washed several times with decreasing salt concentrations, finally 0.1 M sodium phosphate buffer (pH 7.2), 0.5 % SDS, 65°C. After two incubations in RNase A (5 µg/ml) in 0.05 M sodium acetate (pH 7) at room temperature for 3 min each, the gel was dried and autoradiographed for 0.5 to 7 days. Before rehybridizing a gel with another probe it was incubated in the denaturing and neutralizing solutions as described above.

**Northern transfer and slot analysis.** Total RNA was isolated from the brains of normal and scrapie-infected hamsters using a guanidinium/LiCl method (Cathala et al., 1983), and poly(A)<sup>+</sup> RNA was selected on oligo(dT) cellulose (Aviv and Leder, 1972). RNA samples were ethanol-precipitated, washed once with absolute ethanol, and dissolved in 50 % formamide, 2.2 M formaldehyde. After the addition of one-fifth volume 10 % Ficoll, 1 % (w/v) bromophenol blue, the samples were electrophoresed through a 1 % agarose gel. The running buffer was 20 mM MOPS (pH 7.0), 1 mM EDTA, 2.2 M formaldehyde. After electrophoresis, the gel was soaked in 20 x SSC, the RNA transferred to nitrocellulose (Thomas, 1980), and the filter processed as described above for Southern analysis, method A.

For slot hybridisation, total RNA of hamster tissues was purified as described above. RNA samples (100 µl) were incubated with 300 µl of a solution of 6.1 M formaldehyde and 10 x SSC at 65°C for 15 min. Nitrocellulose on slot manifold apparatus (Schleicher and Schuell) was prewetted with a mixture of the formaldehyde-SSC solution. Samples were loaded on the slot manifold apparatus and a vacuum applied (Kafatos et al., 1979). The filters were processed as described above.

**S1-nuclease analysis and primer extension.** For S1 analysis the *TaqI* fragment (Figure 2B, position 4 to 385) was 5'-<sup>32</sup>P-labeled to  $6 \times 10^6$  cpm/pmol ends and the strands separated on a 5% non-denaturing acrylamide gel. For primer extension the same fragment was cut with *SacII* and the 35 nucleotide primer (Figure 2B, position 351 to 385) isolated on a 6.5% denaturing acrylamide gel. S1 analysis was as described by Weaver and Weissmann (1979) using 10  $\mu$ g poly (A)<sup>+</sup> RNA from scrapie-infected hamster brain, at 40°C. For primer extension, primer (3 fmol) was hybridized to 2  $\mu$ g poly (A)<sup>+</sup> RNA from scrapie-infected hamster brain or 10  $\mu$ g yeast carrier RNA for 12 h at 35°C in 10  $\mu$ l 80% FAHB (0.04 M PIPES (pH 6.4), 0.4 M NaCl, 1 mM EDTA in 80% formamide). Reverse transcription was done in 20  $\mu$ l 50 mM Tris-HCl, 30 mM KCl, 8 mM MgCl<sub>2</sub>, 6 mM DTT, 0.5 mM of each dNTP and 10 units reverse transcriptase (Boehringer). The samples were incubated successively at room temperature for 30 min., 42°C for 1h and 46°C for 1h.

**In vitro transcription.** Supercoiled pSPPr-7.1 (1 $\mu$ g) was incubated with 25  $\mu$ l HeLa cell nuclear extract (a gift of M. Aebi and H. Hornig) in 50  $\mu$ l final volume as described by Dignam et al. (1983). After 60 min 50  $\mu$ l 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub> and 2  $\mu$ g RNase-free pancreatic DNase were added, and the incubation continued for 15 min at 30°C. The RNA was purified by proteinase K digestion, phenol extraction and ethanol precipitation, and was S1-mapped with the probe described above.

**Cloning of nucleic acids conjectured to be present in highly purified preparations of scrapie agent.** Purified infectious preparations (Prusiner et al., 1984) were treated with micrococcal nuclease followed by zinc nitrate at 65°C (Butzow and Eichhorn, 1975). In order to extract nucleic acids, SDS was added to 1% and 50 ng rabbit  $\beta$ -globin RNA synthesized *in vitro* with SP6 polymerase using as a template pR $\beta$ G/LIVS24 (Padgett et al., 1985) into which a *NotI* linker (CAGCGGCCGCTG) had been inserted into the *Bam*HI site. The mixture was then heated to 100°C for 5 min and digested with proteinase K (0.3 mg/ml final concentration) for 1 h at

37°C. After phenol, phenol/chloroform and ether extraction, nucleic acids were precipitated with ethanol. First strand synthesis was done in 20 µl as described above for primer extension, except that random primer made from salmon sperm DNA by DNase I digestion was used. For second strand synthesis 20 µl 40 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 200 mM KCl, 0.5 mM β-NAD was added followed by 20 units DNA polymerase I, 0.2 units RNase H and 4 units E. coli DNA ligase. Incubations were at 12°C and 20°C 1 h each. The synthesized cDNA was then phenol extracted and ethanol precipitated. To generate blunt ends the cDNA was treated with 5 units T4 polymerase at 16°C for 30 min in the buffer recommended by the manufacturer (Biolabs) and purified again by phenol extraction and ethanol precipitation. 10 pmole EcoRI linkers (Biolabs) were ligated to the cDNA in 10 µl according to standard protocols (Maniatis et al., 1982), the ligase then inactivated at 65°C for 15 min followed by EcoRI digestion (50 units) for 2 h at 37°C. The linker was separated from the cDNA on an 1% low melting agarose gel. The cDNA was extracted from the agarose in the presence of 2 µg λ DNA, ethanol precipitated, then ligated in 10 µl and packaged as described (Grosveld et al., 1981). The library was amplified on E. coli LE 392 as a plate lysate (Arber et al., 1983). λ DNA was purified from liquid lysates (Arber et al., 1983), inserts were cut out with EcoRI, size selected (100-500 bp) on an 1.2% agarose gel and religated into λgt11/amp3 (Kemp et al., 1983). After packaging the phages were plated on LE 392 at 500 plaques per 8 cm dish in the presence of 0.3 mM IPTG and 0.03% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and then transferred to filters (PALL Bodyne) which were hybridized with nick translated fragment containing the globin sequences of pRβG/LIVS24. Plaques that did not show hybridization to globin or blue color were picked and grown up as lysogens in E. coli BTA 282 (Kemp et al., 1983).

#### **Analysis of λ clones not hybridizing to globin sequences.**

λ DNA was prepared directly from the bacteria previously induced for phage replication. Briefly, lysogens were grown at 32°C overnight, diluted 5x into 25 ml fresh Luria broth and left for another 2 h at 32°C. Induction for phage growth was at 45°C for 15 min and the

cultures were then further grown at 38°C for 2-4 h. Aliquots were checked for lysis upon addition of chloroform. As soon as lysis occurred the cultures were centrifuged, the bacteria resuspended in 1 ml TMG (Arber et al., 1983) and lysed by addition of chloroform for 30 min at room temperature. The phage lysate was incubated with DNAase I (40 µg/ml final concentration) and RNAase A (0.1 mg/ml final concentration) for 30 min at room temperature, spun for 10 min in an Eppendorf centrifuge, the supernatant made 50 mM in EDTA and 0.5% in SDS and then phenol extracted twice. λ DNA was precipitated by the addition of 1 volume isopropanol. The amount of phage DNA obtained was estimated after electrophoresis through a 0.7% agarose gel. Approximately 2-5 µg DNA were digested with MluI and the fragment containing the cDNA insert was purified from a 0.8% low melting agarose gel, if the insert was judged to be larger than 100 bp. The isolated fragments were nicktranslated to a specific activity of  $5 \times 10^7$  to  $10^8$  cpm/µg and hybridized to the following Southern blots at  $5 \times 10^6$  cpm/ml: Hamster genomic DNA was cleaved with EcoRI, electrophoresed through a 0.8% agarose gel (5 µg per lane) and transferred to PALL Biotodyne membrane filters. The blot was cut in strips which were used for individual hybridizations. The same procedure was applied to E. coli LE 392 DNA cleaved with EcoRI (3.3 µg per lane), pRβG/LIVS24 plasmid cleaved with HindIII, BamHI and EcoRI (10 ng per lane) and λgt11/amp3 DNA cleaved with MluI (83 pg per lane together with 0.1 µg sonicated salmon sperm DNA). Hybridizations were in 5xSET, 0.2% SDS, 0.3 mg/ml NaOH-treated salmon sperm DNA, 0.2 mg/ml yeast carrier RNA and 0.1 mg/ml BSA overnight at 65°C. The filters were washed in 0.5xSSC, 0.02% SDS at 60°C and exposed to Fuji RX films at -70°C with intensifier screens.

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## Curriculum Vitae

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|           |                                                                                                                                                                                                          |
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| 1963-1969 | Primarschule in Warth, Thurgau                                                                                                                                                                           |
| 1969-1976 | Besuch der Mittelschule in Sarnen<br>Abschluss mit der eidgenössischen Matura, Typ B                                                                                                                     |
| 1976-1981 | Biochemiestudium an der ETH Zürich an der Abteilung<br>für Naturwissenschaften (X. Bd)                                                                                                                   |
| 1979-1980 | Aufenthalt an der Universität von Rom, Istituto di<br>Chimica Biologica bei Prof. P. Fasella; Ausführung<br>praktischer Arbeit biochemisch-physikalischer Art in<br>Zusammenarbeit mit Prof. A. Bianconi |
| 1981      | Abschluss an der ETH Zürich mit dem Diplom für<br>Naturwissenschaften                                                                                                                                    |
| 1981-1982 | Assistent am Friedrich Miescher Laboratorium der<br>Max-Planck-Gesellschaft in Tübingen. Untersuchung<br>von Adhäsionsproteinen mittels monoklonaler<br>Antikörper im Labor von Dr. W. Birchmeier        |
| 1982-1986 | Doktorarbeit am Institut für Molekularbiologie I der<br>Universität Zürich unter der Leitung von Prof. Dr. C.<br>Weissmann                                                                               |

Ich besuchte die Kurse und Vorlesung folgender Dozenten:

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| Mathematik/Physik: | Meier; Kneubühl;                                                     |
| Chemie:            | Geier; Eschenmoser; Arigoni; Fringeli; Müller;<br>Simon; Wild;       |
| Botanik/Zoologie:  | Ruch; Matile; Kern; Camenzind; Eppenberger;<br>Moor; Bättig; Koller; |
| Genetik:           | Würgler; Hütter;                                                     |
| Mikrobiologie:     | Ettlinger; Leisinger; Hüttér;                                        |
| Molekularbiologie: | Schwyzer; Dutler; Wüthrich;                                          |
| Biochemie:         | Carafoli; Semenza; Winterhalter; Cherry;<br>Gazotti;                 |

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